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Targeting Microglia Necroptosis to Arrest Neuroinflammation

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Abstract

Necroptosis is a caspase-independent form of regulated cell death executed via activation of receptor interacting protein 1 (RIP1) and RIP3, being negatively regulated by caspase-8. Neurodegenerative diseases are hereditary or sporadic conditions characterized by progressive cell death in selected areas of the nervous system, along with oxidative stress, mitochondrial dysfunction and neuroinflammation. Importantly, recent studies have demonstrated that necroptosis is involved in the pathogenesis of several disorders, including neurodegenerative diseases.

Neuroinflammation, in turn, is a response to eliminate the original insult and damaged or dead cells. However, if tissue physiology is not restored, inflammation becomes a chronic condition, where microglia plays a major role. Studies have shown that defects in caspase-8 activation in microglia may promote inflammation through activation of necroptosis.

In the present study, we used the murine BV2 microglia cell line as an *in vitro* model to screen a library of novel compounds, potential modulators of necroptosis, and evaluate if necroptosis modulation may contribute to attenuate microglia-mediated inflammation. Importantly, we identified one hit Oxa12 that inhibits this type of regulated cell death by preventing all necroptosis-associated events, including RIP1/RIP3 necrosome assembly, and MLKL phosphorylation. In addition, Oxa12 decreased TNF-α gene expression and secretion as well as JNK and p38 MAPK activation, while IκB phosphorylation was increased.

In conclusion, we established a robust *in vitro* model of microglia necroptosis and identified a strong inhibitor of this regulated form of cell death that might be a promising candidate molecule for targeting RIP1/3-driven pathologies.

Keywords: Drug screening; Microglia; Molecular targeting; Necroptosis; Neuroinflammation; Small molecules

Resumo

A necroptose é um tipo de morte celular regulada e independente de caspases, que ocorre quando há ativação das proteínas *receptor interacting protein 1* (RIP1) e RIP3, sendo inibida pela caspase-8. As doenças neurodegenerativas são condições hereditárias ou esporádicas caracterizadas pela ocorrência de morte celular progressiva em áreas específicas do sistema nervoso, assim como pela existência de stress oxidativo, disfunção mitocondrial e neuroinflamação. É importante referir que estudos recentes têm demonstrado que a morte celular por necroptose está envolvida na patogénese de várias doenças, incluindo as doenças neurodegenerativas.

A neuroinflamação, por sua vez, visa eliminar tanto a causa inicial do dano celular, como as células mortas que resultam do insulto original. Contudo, se a fisiologia normal do tecido não for restaurada, a inflamação torna-se uma condição crónica. No sistema nervoso central (CNS), são as células da microglia que constituem a primeira linha de defesa, desempenhando um papel crucial na neuroinflamação, através da contínua produção de citoquinas pró-inflamatórias e espécies reativas de oxigénio, entre outros. Estes mediadores inflamatórios podem, por sua vez, ter efeitos neurotóxicos. De facto, a comunicação entre a microglia e os neurónios pode amplificar os sinais inflamatórios e contribuir para a patogénese das doenças neurodegenerativas. De salientar que, tal como descrito recentemente, a inativação de caspase-8 na microglia pode promover inflamação através da ativação da necroptose.

No presente estudo, foi utilizada a linha celular de microglia de murino, BV2, como um modelo *in vitro* para o estudo de duas bibliotecas de novos compostos, potencialmente moduladores da necroptose. Para além disso, avaliámos se a modulação da necroptose pode contribuir para atenuar a inflamação mediada pela microglia.

Em primeiro lugar, otimizou-se o modelo celular. Os resultados obtidos demonstraram que a incubação de células BV2 com o inibidor de caspases, zVAD-fmk,

resultava em níveis elevados de morte celular por necroptose, tal como avaliado através de ensaios de viabilidade e citotoxicidade. Os resultados foram confirmados por *Western blot*, onde observámos um enriquecimento das proteínas RIP1 e RIP3 no proteoma insolúvel das células. Esta estrutura amiloide, ou necrossoma, desempenha um papel preponderante na ativação da necroptose. Pelo contrário, a incubação das células com o inibidor seletivo da atividade cinase da proteína RIP1, necrostatina-1 (Nec-1), reverteu totalmente a necroptose para níveis controlo.

Após a otimização do modelo celular, testámos duas bibliotecas de compostos de famílias diferentes, onde identificámos a molécula Oxa12 como sendo capaz de inibir a necroptose na linha celular BV2. De facto, este composto reduziu os níveis de morte celular induzida por zVAD-fmk, em cerca de 80% ($p < 0.05$). Mais ainda, o composto Oxa12 reprimiu todos os eventos associados à necroptose, incluindo a formação do necrossoma e, também, a fosforilação do resíduo de serina 358 da proteína MLKL pela proteína RIP3. É de salientar que a fosforilação da MLKL neste resíduo induz a sua oligomerização e consequente migração para a membrana plasmática, onde promove a rutura da mesma, sendo por isso um evento crítico na execução da necroptose. Podemos, assim, concluir que o composto Oxa12 é um inibidor forte da necroptose. De forma a melhor caracterizar a atividade do composto identificado, determinámos a concentração à qual tem metade da sua atividade máxima (EC_{50}), assim como a sua toxicidade. Os resultados obtidos demonstraram que o composto Oxa12 inibe a necroptose com um EC_{50} de 1,422 μM, revelando o seu potencial, mesmo a concentrações reduzidas. Para além disso, verificámos que este composto não induz toxicidade em células BV2 controlo, ou seja, sem qualquer tratamento, mesmo a elevadas concentrações.

A necroptose é considerada uma forma de morte celular do tipo inflamatório, devido à libertação dos constituintes intracelulares para o espaço extracelular onde se encontram células imunitárias, tais como macrófagos, que podem potenciar a inflamação.

Desta forma, a nossa hipótese foi a de que o composto Oxa12, ao inibir a necroptose, contribui para uma diminuição da inflamação. Assim, para além dos níveis dos transcritos de vários mediadores inflamatórios, tais como TNF-α, IL-1β, COX2 e NLRP3, avaliados por Real-Time PCR (RT-PCR), determinámos também os níveis de secreção de TNF-α, através da utilização de um ensaio de ELISA. Uma vez que a microglia constitui a principal linha de defesa do CNS, é bem conhecida a sua capacidade de produzir e libertar várias moléculas inflamatórias. Os resultados obtidos demonstraram que a incubação das células BV2 com zVAD-fmk resulta num aumento da transcrição dos genes TNF-α e IL-1β, assim como dos níveis de TNF-α secretados. Pelo contrário, a coincubação com Nec-1 foi capaz de reverter totalmente aqueles parâmetros. De particular importância é o facto do composto em estudo, Oxa12, ter diminuído os níveis de expressão e libertação de TNF-α.

De forma a determinar quais as vias de sinalização especificamente moduladas pelo Oxa12, avaliámos os níveis de fosforilação de resíduos específicos das proteínas JNK (Treonina 183 e Tirosina 185) e p38 (Treonina 180 e Tirosina 182), componentes chave de duas vias de sinalização da família das MAP cinases. Os resultados revelaram um aumento significativo da fosforilação da proteína JNK, indicativos de ativação desta via, após incubação das células com zVAD-fmk, bem como a concomitante diminuição após adição de Nec-1 ou Oxa12. Estes resultados estão de acordo com os de outros autores, que referem a ativação da proteína JNK como um fator preponderante na indução da necroptose, nomeadamente promovendo a produção autócrina de TNF-α. Relativamente à proteína p38, a sua função na necroptose é ainda pouco conhecida, para além de contraditória. Enquanto alguns autores defendem que esta via de sinalização não interfere no mecanismo de morte, outros demonstraram que a inibição da necroptose induzida por TNF-α resulta na sua ativação. Curiosamente, os resultados obtidos neste trabalho revelaram um aumento dos níveis de p38 fosforilada após incubação das células com zVAD-fmk e, por sua vez, uma diminuição com Nec-1 ou Oxa12. É possível que este aumento de ativação com zVAD-fmk

esteja relacionado com a indução de respostas inflamatórias, uma vez que tanto a via da JNK como da p38 estão envolvidas na inflamação.

Para além das vias de sinalização mediadas pela família das MAP cinases, avaliámos também os níveis de fosforilação da proteína Akt. Estudos recentes demonstraram que a inibição da proteína Akt é protetora num modelo de necroptose induzida por TNF-α em células L929. No entanto, outros estudos descrevem que o tratamento celular apenas com zVAD-fmk não é suficiente para induzir a fosforilação e, consequente, ativação da proteína Akt, sendo necessária a atividade conjunta de TNF-α e zVAD-fmk. Por outro lado, outros autores demonstraram que o zVAD-fmk é capaz de induzir a ativação da proteína Akt, sendo essa ativação dependente especificamente da fosforilação do resíduo Treonina 308. No nosso estudo, usámos um anticorpo especifico para a fosforilação do resíduo Serina 473, o que poderá justificar a ausência de diferenças significativas entre as condições estudadas. Por fim, fomos avaliar a ativação do fator de transcrição NF-κB, indiretamente, através dos níveis de fosforilação do seu inibidor IκB. Os nossos resultados demonstraram uma diminuição dos níveis de IκB fosforilado em células BV2 tratadas com zVAD-fmk e um aumento concomitante após adição de Nec-1 e Oxa12, sugerindo uma ativação daquele fator de transcrição. Estes resultados estão de acordo com os obtidos em estudos anteriores, confirmando que a via de sinalização mediada pelo NF-κB não possui um papel preponderante na ativação da necroptose ou na expressão de TNF-α. Para além disso, é ainda possível que o composto Oxa12 promova um desvio da sinalização da necroptose para vias de sobrevivência celular mediadas pelo NF-κB. No entanto, são necessários mais estudos que confirmem esta hipótese.

Em conclusão, estabelecemos um modelo celular robusto de necroptose em microglia e identificámos um forte inibidor químico deste tipo de morte celular regulada. O composto Oxa12 é um candidato promissor para ser utilizado na terapêutica de patologias associadas à ativação das proteínas RIP1 e RIP3.

Palavras-chave: Alvos moleculares; Microglia; Necroptose; Neuroinflamação; Pequenas moléculas; Pesquisa de fármacos

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Abbreviations

ABL – abelson leukemia

AD – Alzheimer's disease

ALS – amyotrophic lateral sclerosis

ANOVA – analysis of variance

AP1 – activating protein 1

ASC – apoptosis-associated speck-like protein containing CARD

Aβ – amyloid-β

BCR – breakpoint cluster region

CD – cluster of differentiation

cFLIP – cellular FLICE-like inhibitory protein

cIAP – cellular inhibitor of apoptosis

CNS – central nervous system

COX2 – cyclooxygenase 2

CYLD – cylindromatosis

DAMPs – damage-associated molecular patterns

ELISA – enzyme linked immunosorbent assay

ERK – extracellular signal-regulated kinase

FADD – FAS-associated death domain protein

HD – Huntington's disease

HMGB1 – high-mobility group box 1

HOIL1 – hem-oxidized iron-regulatory protein 2 ubiquitin ligase-1

HOIP – HOIL-1 interacting protein

IFNs – interferons

IKK – inhibitor of κB kinase

IL – interleukin

iNOS – inducible nitric oxide synthase

IRAK4 – IL-1R-associated kinase 4

IκB – inhibitor of κB

JNK – c-Jun N-terminal kinase

LDH – lactate dehydrogenase

LPS – lipopolysaccharide

LUBAC – linear ubiquitin chain assembly complex

MAPKs – mitogen-activated protein kinases

MCMV – murine cytomegalovirus

MCP1 – monocyte chemoattractant protein 1

MLKL – mixed lineage kinase like

MS – multiple sclerosis

mTOR – mechanistic target of rapamycin

MyD88 – myeloid differentiation primary response 88

Nec-1 – necrostatin-1

NEMO – nuclear factor-κB essential modulator

NF-κB – nuclear factor-κB

NLRP3 – NLR protein pyrin domain containing 3

NLRs – nucleotide-oligomerization binding domain proteins

NO – nitric oxide

NSA – necrosulfonamide

PAK1 – serine/threonine protein kinase 1

PAMPs – pathogen associated molecular patterns

PCD – programmed cell death

PD – Parkinson's disease

PGAM5 – phosphoglycerate mutase family member 5

PI – propidium iodide

PINK1 – PTEN-induced putative kinase 1

PKC – protein kinase C

PRR – pattern recognition receptors

PS – phosphatidylserine

qRT-PCR – quantitative real-time PCR

RAGE – receptor for advanced glycation end-products

RHIM – RIP homotypic interaction motif

RIP – receptor-interacting protein

ROS – reactive oxygen species

SEM – standard error of the mean

SHARPIN – SHANK-associated RH domain-interacting protein

SIRS – systemic inflammatory response syndrome

SP1 – specificity protein 1

TAK1 – transforming growth factor beta-activated kinase 1

TGF-β – transforming growth factor β

TLR – toll-like receptor

TNFR – TNF-α receptor

TNF-α – tumor necrosis factor α

TRADD – TNFR-associated death domain

TRAF2 – TNFR-associated factor 2

TRAIL-R – TNF-related apoptosis-inducing ligand receptor

TRIF – Toll-IL-1 receptor domain-containing adaptor inducing IFN-β

TWEAK – TNF-related weak induced of apoptosis

XIAP – X-linked IAP

zVAD-fmk – Z-Val-Ala-Asp-fluoromethylketone

α-syn – α-synuclein

I. INTRODUCTION AND OBJECTIVES

Dementia is a clinical syndrome characterized by progressive deterioration in cognitive ability and capacity for independent living (Prince M et al, 2013). The global incidence of dementia places a considerable burden on society, since overall life expectancy has greatly increased. Indeed, aging populations worldwide face a growing problem of neurodegenerative diseases (Organization WH, 2012).

1. Neurodegenerative diseases

Neurodegenerative diseases are defined as hereditary or sporadic conditions characterized by progressive nervous system dysfunction. However, only an extremely small proportion (less than 5%) are caused by genetic mutations, with the remaining proportion being sporadic. These disorders are often associated with atrophy of the affected central or peripheral structures of the nervous system and include diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS), but also epilepsy, stroke and others. AD, PD and ALS are the major diseases in terms of mortality, morbidity, and health care costs. However, their triggering mechanisms are far from fully deciphered, and effective diagnosis and treatment are still highly demanded.

Overall, the hallmarks of neurodegenerative diseases include cellular death in selected areas of the nervous system, as well as abnormal protein assemblies, oxidative stress, mitochondrial dysfunction and neuroinflammation.

2. Neuroinflammation

In addition to neurodegeneration, also neuroinflammation has been implicated in many central nervous system (CNS) diseases including acute brain damage and chronic

neurodegenerative disorders (Banati RB et al, 1998; Raine CS, 1994). Neuroinflammation is a complex cellular and molecular response to stress that attempts to contain the injury by way of clearing pathogens, dead or damaged host cells, and aid in returning the damaged area to its normal state. If tissue physiology is not restored, inflammation becomes a chronic condition. Of note, a characteristic feature of chronic inflamed tissues is the presence of an increased number of monocytes, as well as monocyte-derived tissue macrophages, called microglia cells in the CNS, in addition to an increased expression of acute phase proteins and proinflammatory cytokines which are hardly evident in the normal brain (Rubio-Perez JM et al, 2012). Microglia, astrocytes, neurons and the complementary system are all involved in the inflammatory reaction in the CNS. Although it was previously thought that the CNS was an immune-privileged site, due to its isolation from the immune system by the blood–brain barrier, the lack of draining lymphatics, and the apparent immunoincompetence of microglia, now it is known that certain features of the inflammatory process occur normally in response to injury, infection or disease (Rubio-Perez JM et al, 2012). More specifically, in response to brain injury, an inflammatory response mediated by microglia and astrocytes is induced, thus activating signaling pathways that may lead to neurodegeneration (Meraz-Ríos MA et al, 2013) (Figure 1). Whether brain inflammation is a cause or a consequence of neurodegeneration is still a matter of debate.

2.1. Microglia-mediated neuroinflammation

Microglia are resident brain cells derived from monocyte precursors during embryogenesis. They constitute the main line of the innate immune defense in the CNS (Meraz-Ríos MA et al, 2013). In humans, microglia constitute up to 16% of the CNS cellular population, where they play a central role in neuroinflammation (Norden DM et al, 2013).

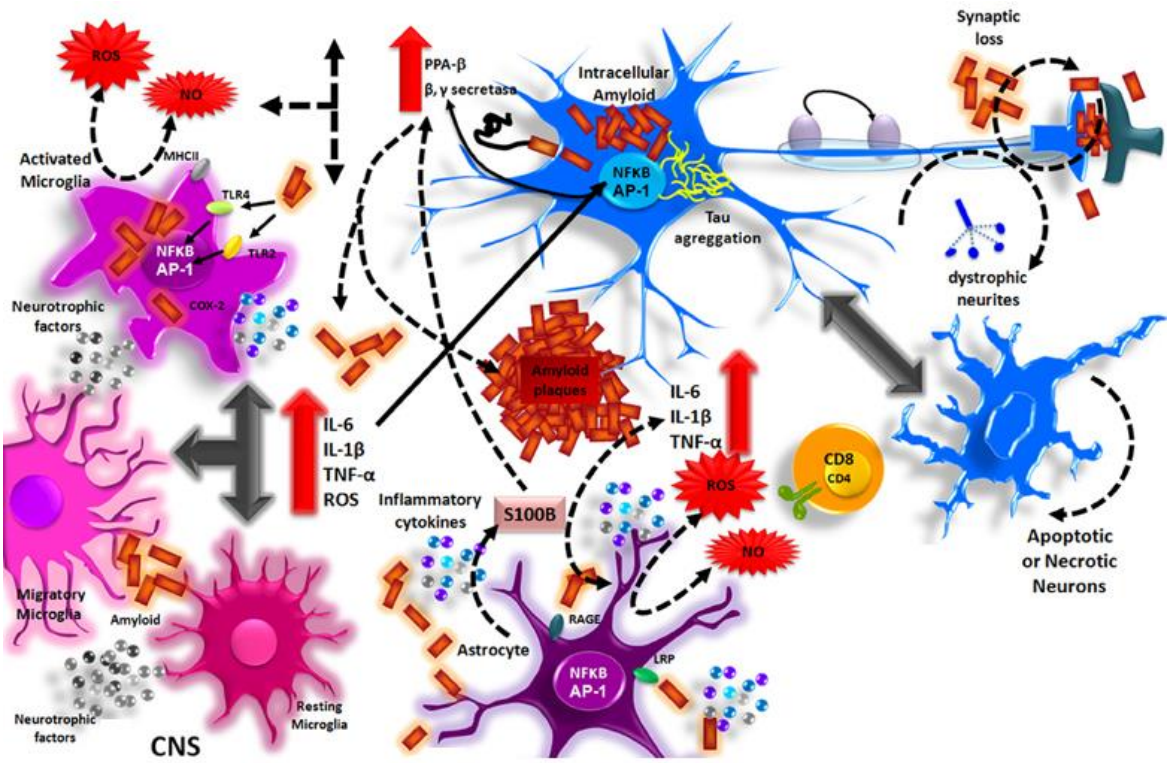

Figure 1. Neuroinflammation in AD. Amyloid-β (Aβ) aggregates activate microglia through toll-like receptors (TLRs) and receptors for advanced glycosylation end products (RAGE). These receptors, in turn, activate nuclear factor-κB (NF-κB) transcription factor, which induces reactive oxygen species (ROS) production and expression of proinflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α). These inflammatory factors act on neurons and stimulate astrocytes, which amplify proinflammatory signals, inducing neurotoxic effects. Adapted from Meraz-Ríos MA et al, 2013.

Normally, microglia exist in an inactive or resting state, characterized morphologically by a small soma with branching processes (Norden DM et al, 2013). However, some studies have reported that microglia in the healthy CNS are not truly "resting", since they are engaged in environment surveillance, constantly sampling areas around them in efforts to maintain homeostasis (Nimmerjahn A et al, 2005). Under pathological conditions, such as neurodegenerative diseases, these cells become activated,

migrate, surround damaged or dead cells, and subsequently clear cellular debris from the injured area (Rubio-Perez JM et al, 2011).

Microglia possess several families of pattern recognition receptors (PRR), including specific toll-like receptors (TLRs) and receptors for advanced glycosylation end products (RAGE) that upon binding of conserved motifs of microbial and viral-derived molecules classified as pathogen-associated molecular patterns (PAMPs), activate downstream signaling cascades that culminate in microglial activation. In addition, several of these receptors are also involved in the recognition of distinct molecules released from endogenous compartments or modified in structure, such as abnormal protein aggregates, that constitute damage-associated molecular patterns (DAMPs) (Landreth GE et al, 2009; Heneka MT et al, 2014). Upon activation, microglia suffer visible morphological changes, with acquisition of an amoeboid form consisting in decreased branching and soma growth, and expression of a wide variety of specific cellular surface receptors and key enzymes, presenting increased phagocytic capabilities and secretion of inflammation-related molecules (Cherry JD et al, 2014). In the brain, this inflammatory response is fundamental to protect the CNS. However, uncontrolled or prolonged neuroinflammation is potentially harmful and can result in cellular damage, thus contributing to neurodegeneration (Frank-Cannin TC et al, 2009).

Several studies have led to the concept that, phenotypically, microglia can present different activation stages, ranging from “classical” activation (M1 activation) to “alternative activation” (M2 activation) (Cherry JD et al, 2014). Classically activated microglia are most commonly associated with disease states that are driven by inflammation, such as chronic inflammatory diseases, and leads to increased release of reactive oxygen species (ROS) and nitric oxide (NO), as well as production of proinflammatory mediators such as cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor α (TNF-α), which in turn are responsible for autocrine and paracrine inflammatory signaling in other glial cells and

neurons. Additionally, there is also increased production of chemokines such as IL-8 and monocyte chemoattractant protein 1 (MCP1), which can recruit other microglia and peripheral macrophages to the injury site. In contrast, alternative activation states are associated with protection from diseases and are classically stimulated by IL-4 and IL-13, being characterized by the repression of proinflammatory gene expression and increased production of neurotrophic factors and anti-inflammatory cytokines such as IL-10, transforming growth factor β (TGF-β), IL-4 and IL-13. This phenotype reduces inflammation, promotes phagocytosis and potentiates tissue repair (Lyman M et al, 2013; Meraz-Ríos MA et al, 2013).

Microglia activation by abnormal protein aggregates, such as amyloid-β (Aβ) and α-synuclein (α-syn) aggregates, or by lipopolysaccharide (LPS), or even injury, promotes the stimulation of nuclear factor-κB (NF-κB)-dependent pathway that, in turn, is required for cytokine production. The subsequent activation of mitogen-activated protein kinase (MAPK) pathways may induce proinflammatory gene expression leading to the production of proinflammatory cytokines, chemokines and ROS (Rubio-Perez JM et al, 2011). Indeed, several studies have shown that the three MAPK pathways, extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and p38 are activated in stimulated-microglia, thus contributing to TNF-α and IL-1β production (Kim EK et al, 2010). Conversely, others have demonstrated that microglia-induced production of ROS and proinflammatory cytokines can also stimulate MAPK signaling pathways, constituting a positive feedback loop (Kim EK et al, 2010). The proinflammatory mediators produced by classically activated microglia can then activate astrocytes and the products released by both cell types may exert neurotoxic effects. Therefore, communication between microglia and astrocytes and/or neurons can amplify the proinflammatory signals and contribute to the pathobiology of neurodegenerative diseases (Saijo K et al, 2011) (Figure 2). In fact, long-term inflammation can have disastrous consequences in the CNS, ranging from loss of synapses to impaired

cognition and overt neurodegeneration (Rao JS et al, 2012; Hein AM et al, 2009). In this regard, the therapeutic modulation of microglia-induced inflammation in the CNS may be a useful approach to prevent or attenuate disease progression.

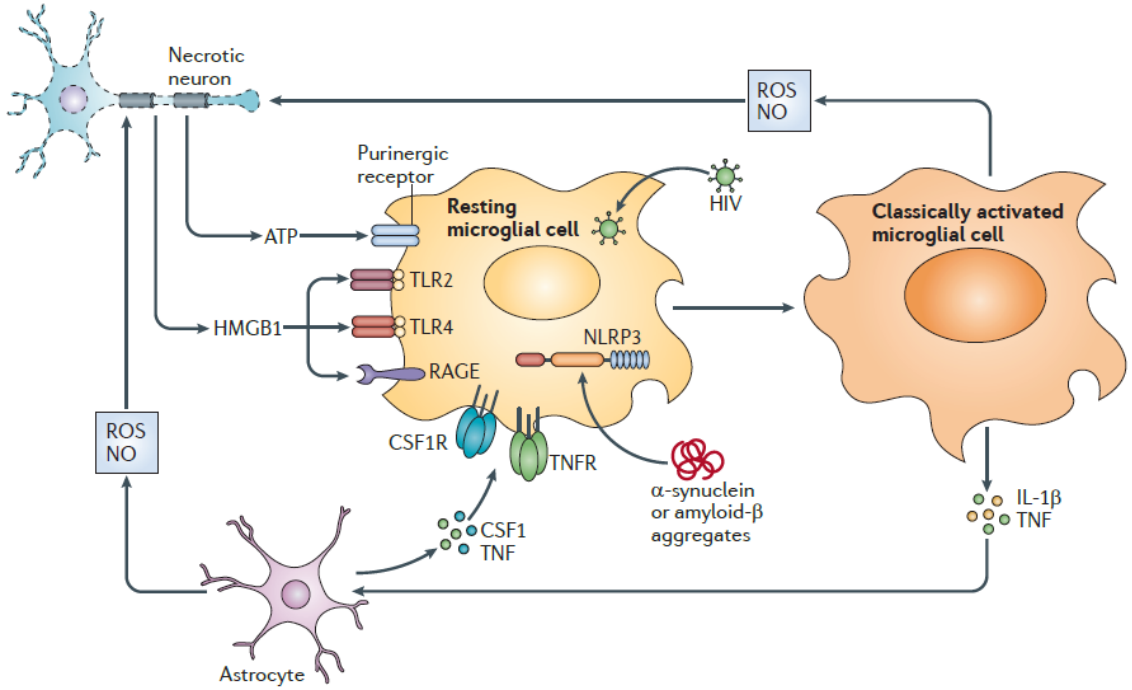

Figure 2. Classically activated microglia in neurodegenerative diseases. Several disease-associated factors such as DAMPs and neurodegenerative disease-specific protein aggregates activate microglia through PRR to establish an M1-like microglial cell phenotype. Proinflammatory mediators produced by classically activated microglia activate astrocytes, and the products released by activated microglia and astrocytes may exert neurotoxic effects. Communication between microglia and astrocytes and/or neurons may therefore amplify proinflammatory signals initially sensed by microglia, and contribute to pathology of neurodegenerative diseases (Saijo K et al, 2011).

2.2. Inflammatory mediators

2.2.1. Cytokines

Cytokines are a group of small and nonstructural soluble proteins with molecular weights ranging from 8 to 40 kDa secreted not only by a variety of immune cells but also by

nonimmune cells (e.g., Schwann cells or fibroblasts) (Rubio-Perez JM et al, 2012). Cytokines include TNFs, ILs and interferons (IFNs), among other molecules. These molecules play an important role in CNS development during embryogenesis, being involved in the stimulation and inhibition of cell growth and differentiation, apoptosis, antiviral activity, upregulation of surface membrane proteins and inflammatory responses (Meraz-Ríos MA et al, 2013; Rubio-Perez JM et al, 2012). However, they are also involved in the pathogenesis of several disorders, especially neurodegenerative diseases (Meraz-Ríos MA et al, 2013). Indeed, elevated levels of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α have been reported in mouse models of AD, as well as in PD, ALS and MS patients, where they contribute to neurodegeneration (Jiang H et al, 2011; Patel NS et al, 2005).

In other cases, proinflammatory cytokines might favor microglial and astrocytic activation to ultimately promote phagocytosis and neuronal survival. Nevertheless, chronic cytokine production is strongly related to neurodegeneration and strategies to tackle neuroinflammation are needed, possibly to be used as a therapeutic approach to attenuate neuronal death.

TNF-α

TNF-α is a pleiotropic cytokine that is present at increased levels in several neurodegenerative diseases (Montgomery SL et al, 2012; Liu S et al, 2014). It plays a central role in initiating and regulating the cytokine cascade during the inflammatory response (Rubio-Perez JM et al, 2012). Although astrocytes and neurons are able to produce TNF-α, it is assumed that microglia cells are the major source of this cytokine during neuroinflammation. However, since activated microglia may trigger different signaling pathways, including NF-κB and MAPKs, depending on the inflammatory stimuli, it is difficult to determine which pathway is indeed implicated in the induction of TNF-α expression (Olmos G et al, 2014). Moreover, TNF-α secreted by glial cells can activate them in an

autocrine manner and sustain cytokine production and astrogliosis. TNF-α is also involved in other cellular responses besides inflammation, including apoptosis and necrosis (Liu S et al, 2014). Curiously, a recent study on MS showed that TNF-α released by necroptotic microglia is able to further induce necroptosis in oligodendrocytes *in vitro*, thus contributing to disease progression (Ofengeim D et al, 2015).

IL-1

The IL-1 family is a group of 11 cytokines basally expressed in the healthy CNS. IL-1α and IL-1β are the most studied members, because they were first discovered and also for their strong proinflammatory effects. Microglia and astrocytes are the primary source of IL-1β in the CNS (Toda Y et al, 2002); however, oligodendrocytes and neurons can also synthetize IL-1β and its signaling receptor (IL-1R) (Fogal B et al, 2008).

IL-1β is implicated in the progression of chronic neurodegenerative diseases, such as AD and PD. In fact, this cytokine increases rapidly in response to stress or pathogenic invasion of the CNS (Rothwell NJ et al, 2000). Once secreted, IL-1β can stimulate its own production in an autocrine/paracrine manner and increase the expression of other relevant cytokines and proteins, such as IL-6, TNF-α and S100B (Kim SH et al, 2004). IL-1β has been reported to play an important role in neuronal degeneration through S100B present in reactive astrocytes. IL-1β also induces ROS and IL-6 production in astrocytes, thus stimulating inducible nitric oxide synthase (iNOS) activity that culminates in neuroinflammation and neuronal damage (Meraz-Ríos MA et al, 2013; Rubio-Perez JM et al, 2012).

IL-6

IL-6 is a potent pleiotropic cytokine mainly produced by activated microglia in different brain regions that can induce a strong inflammatory response (Lyman M et al,

2013). Importantly, significant high levels of IL-6 were found in patients with AD, PD, and MS. Like IL-1β, IL-6 can also stimulate the release of other proinflammatory cytokines and acute phase proteins, in microglia and astrocytes (Meraz-Ríos MA et al, 2013). Finally, this cytokine also plays a complex role in regulating cognitive function (Yirmiya R et al, 2011), since increased levels of IL-6 were associated with age-related cognitive decline (Weaver JD et al, 2002).

2.3. NLRP3 inflammasome

Inflammasomes are multimeric protein complexes that assemble in the cytosol after sensing PAMPs or DAMPs (Lamkanfi M et al, 2014; Martinon F et al, 2002). Activation of the inflammasome is a key function mediated by the innate immune system. The NOD-like receptor (NLR) protein pyrin domain containing 3 (NLRP3) inflammasome is the most extensively studied and it is formed after oligomerization of NLRP3 and subsequent recruitment of adaptor protein apoptosis-associated speck-like protein containing CARD (ASC) and pro-caspase-1 (Takeuchi O et al, 2010). Upon activation of NLRP3, ASC proteins assemble into fiber-like structures culminating in the production of a large protein aggregate that amplifies the activation of caspase-1 (Martinon F et al, 2002; Sutterwala FS et al, 2014). The NLRP3 inflammasome is activated in response to a variety of infectious stimuli or to cellular stress caused by various danger signals, including misfolded protein aggregates and aberrant accumulation of certain metabolites (Takeuchi O et al, 2010).

Importantly, inflammasomes have been linked to several disorders, including neurodegenerative diseases. For instance, it was recently shown that *Nlrp3* gene expression increases in the spinal cord during experimental autoimmune encephalomyelitis progression, an animal model that mimics MS. In contrast, *Nlrp3*-deficient mice present less severe disease, accompanied by a reduction in inflammatory cells infiltration and astrogliosis (Gris D

et al, 2010; Inoue M et al, 2012). Moreover, in AD, fibrillary Aβ induces NLRP3 inflammasome-dependent activation of caspase-1, suggesting a link between inflammasome activation and AD (Halle et al, 2008). Similarly, also fibrillary α-syn fully activates the NLRP3 inflammasome in PD by inducing caspase-1 activation and mature IL-1β production (Codolo G et al, 2013).

Other proinflammatory mediators, apart from cytokines and inflammasomes, such as iNOS and cyclooxygenase-2 (COX2) are also involved in the inflammatory response mediated by activated microglia, being highly regulated by diverse adaptor molecules.

3. Neurodegeneration and mechanisms of cell death

Programmed cell death (PCD) include apoptosis and autophagic cell death and it is crucial for normal neural development. PCD regulates the number and types of cells in the developing brain and spinal cord, and plays a key role in constructing an efficient neuronal network (Miura M, 2011). Under pathological conditions, PCD is also responsible for the loss of neurons associated with neurodegenerative diseases (Tendi EA et al, 2010).

Apoptosis is a form of regulated cell death executed by a group of intracellular cysteine proteases, namely caspases (Thornberry NA et al, 1998). This type of cell death occurs by two well-characterized pathways: the extrinsic pathway, which is activated through the binding of various death-inducing ligands to their respective receptors; and the intrinsic pathway that is activated after mitochondrial intermembrane space proteins are released into the cytosol (Du C et al, 2000; Peter ME et al, 2003). Ultimately, apoptosis leads to phosphatidylserine (PS) exposure on the cell surface, nuclear condensation, membrane blebbing and genomic DNA fragmentation (Elmore S, 2007). Importantly, this type of cell death is frequently implicated in neurodegeneration. For instance, studies in the brain of AD

patients showed a strong upregulation of apoptosis when compared to healthy age-matched controls, with activation of caspase-3 being detected in more than 50% of hippocampal neurons (Su JH et al, 2011; Colurso GL et al, 2003). Similarly, in PD, apoptosis is also considered the dominant mechanism for neurodegeneration (Kountouras J et al, 2012).

Conversely, necrosis is characterized by a rapid loss of plasma membrane integrity, in addition to organelle swelling and ensuing inflammatory responses (Festjens N et al, 2006; Vandenabeele P et al, 2010). This type of cell death frequently occurs when cells are challenged with excessive external stress, such as heat, ischemia, and pathogen infection (Zhou W et al, 2014). For a long time, necrosis was considered as an accidental and passive cell death mechanism. Interestingly, the purely unregulated nature of necrosis was questioned in 1988, when some authors reported that distinct cell types died in response to the same trigger, TNF-α, while manifesting either the features of apoptosis or a morphology without nuclear disintegration (Laster SM et al, 1988). Since then, studies have reported that a subset of necrosis, known as necroptosis, may be a form of regulated cell death (Degterev A et al, 2005). Importantly, some studies have shown that necroptosis is involved in the pathogenesis of several diseases, including neuronal damage induced by ischemic insults, as well as Huntington's disease (HD), ALS and MS (Vandenabeele P et al, 2010).

3.1. Necroptosis

During a long time, apoptosis was considered the sole form of regulated cell death during development, homeostasis and disease, whereas necrosis was regarded as an unregulated and uncontrollable process. However, evidence now reveal that some subtypes of necrosis such as necroptosis can also be regulated (Vandenabeele P et al, 2010).

Necroptosis has been described as an alternative cell death pathway triggered by death receptor signaling in multiple cell types (Degterev A et al, 2005). In addition, it was already demonstrated that necroptosis involves not only the early loss of cytoplasmic membrane integrity and then a loss of mitochondrial membrane potential, but also the activation of autophagy, probably as a self-clearance mechanism (Degterev A et al, 2005; Holler N et al, 2000). Nevertheless, necroptotic cell death *in vitro* is confirmed by plasma membrane rupture and lack of specific apoptotic markers such as caspase activation, chromatin condensation or nucleosomal-sized DNA fragmentation. By flow cytometry, necrotic cells can be distinguished by the concomitant inclusion of fluorescent DNA dyes such as propidium iodide (PI) and positive Annexin V staining, whereas live cells and early apoptotic cells are impermeable to PI (Krysko DV et al, 2008).

3.1.1. Necroptosis activation *in vitro*

Necroptosis was first recognized in 1998 as a caspase-independent form of regulated cell death that can be triggered by treatment with TNF-α only in the presence of a pan-caspase inhibitor, such as Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) (Vercammem D et al, 1998). Since then, necroptosis has been intensively studied upon stimulation of death receptors, particularly tumor necrosis factor receptor 1 (TNFR1). However, some studies have shown that the death receptor cluster of differentiation (CD)95 and tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor (TRAIL-R)1/R2 are also inducers of necroptosis (Fouqué A et al, 2014). Currently, it is known that necroptosis can be initiated not only by ligands of the death receptor family but also by a variety of extracellular and intracellular stimuli that induce their expression and/or activation (Upton JW et al, 2012). Throughout the years, several authors have reported that various cell types can be sensitized to death receptor-induced necroptosis by inhibition of caspases with

pharmacological compounds, such as zVAD-fmk. Indeed, the murine fibroblast-like cell line L929 undergo spontaneous or TNF-induced necroptosis after treatment with zVAD-fmk (Vercammen D et al, 1998). Later studies demonstrated that this necroptotic response is, at least in part, mediated via autocrine TNF-α secretion (Wu YT et al, 2011). Importantly, various cellular stimuli can also induce activation of the proinflammatory and cell death protective NF-κB that, when activated, might lead to the production and autocrine secretion of TNF-α, thus enabling autocrine TNF receptor (TNFR) stimulation. In addition, other reports have also shown that T cells are sensitized for TNF-induced, TRAIL-induced or CD95-induced necroptosis by zVAD-fmk (Holler N et al, 2000).

TLRs are key sensors of the innate immune system that can recognize endogenous DAMPs to activate inflammatory signaling. During TLR2, TLR3, TLR4, TLR5 or TLR9 stimulation, caspase-8 inhibition promotes necroptosis that is dependent on adaptor molecules such as myeloid differentiation primary response 88 (MyD88) in macrophages and other cell types (Kaiser WJ et al, 2013; Schworer SA et al, 2014). Importantly, signaling through MyD88 also induces autocrine secretion of TNF-α and further signaling via TNFR (Kaiser WJ et al, 2013). On the other hand, cellular inhibitor of apoptosis protein (cIAP)1/2 antagonist, TLR3 agonists and IFN-γ may trigger necroptosis independently of death receptors (Feoktistova M et al, 2011; Kim SJ et al, 2013).

Murine cytomegalovirus (MCMV) can also activate DNA-dependent activator of interferon regulatory factors (DAI), therefore inducing necroptosis (Upton JW et al, 2012), and finally, a number of cell-intrinsic stimuli, such as genotoxic stress may activate necroptosis by downregulation of IAPs (Tenev T et al, 2011) (Figure 3).

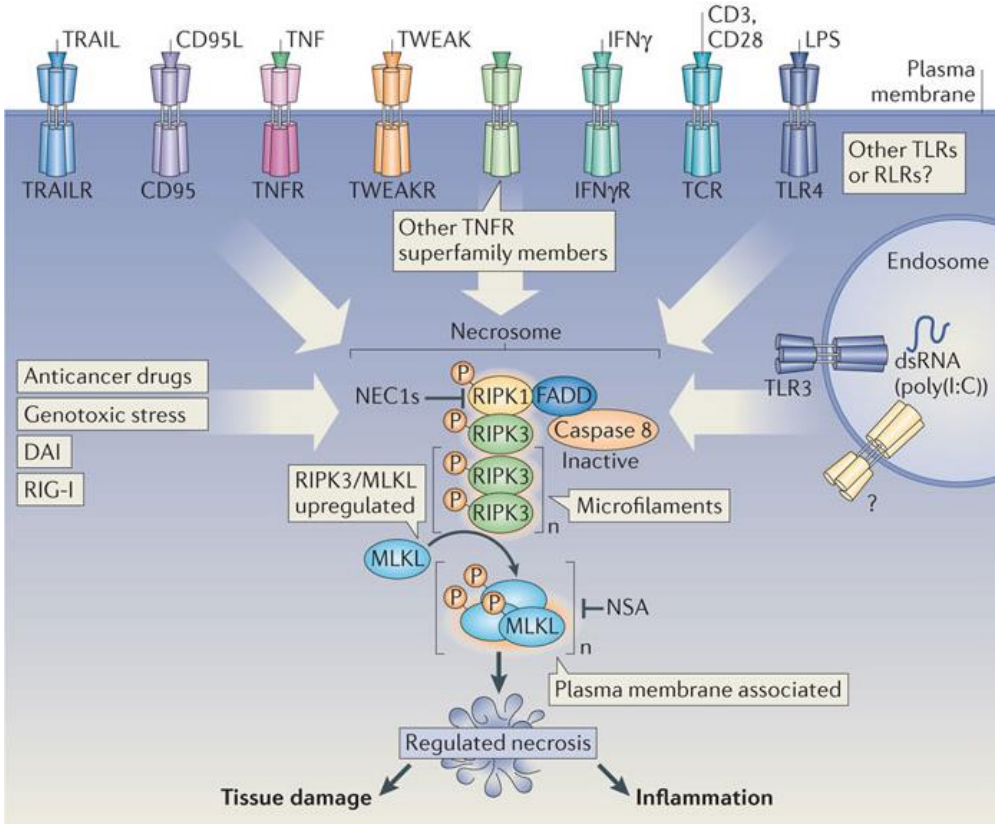

Figure 3. Necroptosis activators. Many triggers of necroptosis have been identified and include death receptors TNFR, CD95, TRAIL, TNF-related weak induced of apoptosis (TWEAK), as well as genotoxic stress and PAMPs, such as LPS. Recently, IFNs were also shown to induce necroptosis (Berghe TV et al, 2014).

3.1.2. Molecular mechanisms of necroptosis

Although there is a large variety of necroptosis activators, the members of the TNFR superfamily, are still the major mediators of this type of cell death. At the molecular level, after TNFR triggering, receptor-interacting protein 1 (RIP1) ubiquitination controls the switching between pro-survival signaling, and apoptotic and/or necroptotic cell death. Briefly, after ligand binding to TNFR1, two complexes with opposing signaling can be formed. The pro-survival complex, also called complex I, contains the adaptor protein TNFR1-associated death domain protein (TRADD), RIP1, TNF-associated factor 2 (TRAF2), E3-ubiquitin ligases

cIAP-1, cIAP2, and hem-oxidized iron-regulatory protein 2 ubiquitin ligase-1 (HOIL-1) that, along with HOIL-1L interacting protein (HOIP) and SHANK-associated RH domain-interacting protein (SHARPIN), form the linear ubiquitin chain assembly complex (LUBAC) (Haas TL et al, 2009) (Figure 4). In complex I, RIP1 is rapidly modified by ubiquitination, which includes Met1-linear and Lys63-linked polyubiquitination mediated by E3 ligases LUBAC and cIAP1, respectively. Ubiquitination of RIP1 functions as a scaffold for the recruitment of NF-κB essential modulator (NEMO) and transforming growth factor beta-activated kinase 1 (TAK1), the critical mediators of NF-κB and MAPK pathways, respectively. In fact, RIP1 recruitment to complex I and its polyubiquitination by TRAF2/5, cIAP1 and cIAP2 promotes the activation of ERK, JNK and p38 signaling pathways, which leads to cell survival and inflammation (Mahoney DJ et al, 2008). Complex I remains associated with TNFR1 (Bertrand MJ et al, 2008). Importantly, RIP1, but not its kinase activity, has an important role in the survival-promoting activity mediated by complex I (Degterev A et al, 2005). By contrast, deubiquitination of RIP1 by cylindromatosis (CYLD), a Lys63-specific deubiquitinating enzyme, promotes apoptosis or necrosis through the formation of the cytoplasmic death-inducing signaling complex termed complex II (Hitomi J et al, 2008) (Figure 4). Endosomal internalization of TNFR1 is accompanied by the release of the complex from TNFR1 and recruitment of caspase-8, FAS-associated death domain protein (FADD), TRADD, and cellular FLICE-like inhibitory protein (cFLIP), resulting in the formation of complex IIa. This complex can trigger apoptosis through activation of downstream caspase-3 and -7, by FADD and caspase-8, and inhibit necrosis by cleavage of RIP1, RIP3, and CYLD (O'Donnell MA et al, 2011). On the other hand, complex IIb initiates necroptosis. If recruitment of caspase-8 is prevented, RIP1 and RIP3 accumulate and become phosphorylated, leading to necroptosis (He S et al, 2009; Zhang DW et al, 2009) (Figure 4). RIP1 and RIP3 kinases contain small protein domains called rip homotypic interaction motifs (RHIMs), by which they interact. Indeed, active RIP1 can move into alternative protein complexes, where it can activate RIP3

via RHIM-RHIM interactions, leading to necroptosis (Sun X et al, 2002). More recently, it was demonstrated that interaction between RIP1 and RIP3 forms a large amyloid-like structure, named necrosome, which is a key event in the necroptotic signaling pathway (Li J et al, 2012). The physical interaction between both proteins allows for their auto- and trans-phosphorylation, which is important for proper necrosome activity (Li J et al, 2012; Ofengeim D et al, 2013). What determines the switch from complex IIa to IIb is still unclear.

Although less explored, necroptosis can also be initiated by members of the PRR family, including TLR that are expressed by cells of the innate immune system, such as microglia cells in the CNS, as previously described. The TLR-activated microglia undergo downstream Toll-IL-1 receptor domain-containing adapter-inducing interferon-β (TRIF) activation, leading to RIP3-dependent necroptosis, when exposed to caspase inhibitors (Fricker M et al, 2013). Indeed, after TLR3/TLR4 stimulation in combination with caspase inhibition, TRIF, which is an adaptor-signaling molecule for receptors possessing a RHIM sequence, interact with RIP1/RIP3 to activate necroptosis (He S et al, 2011; Kaiser WJ et al, 2013).

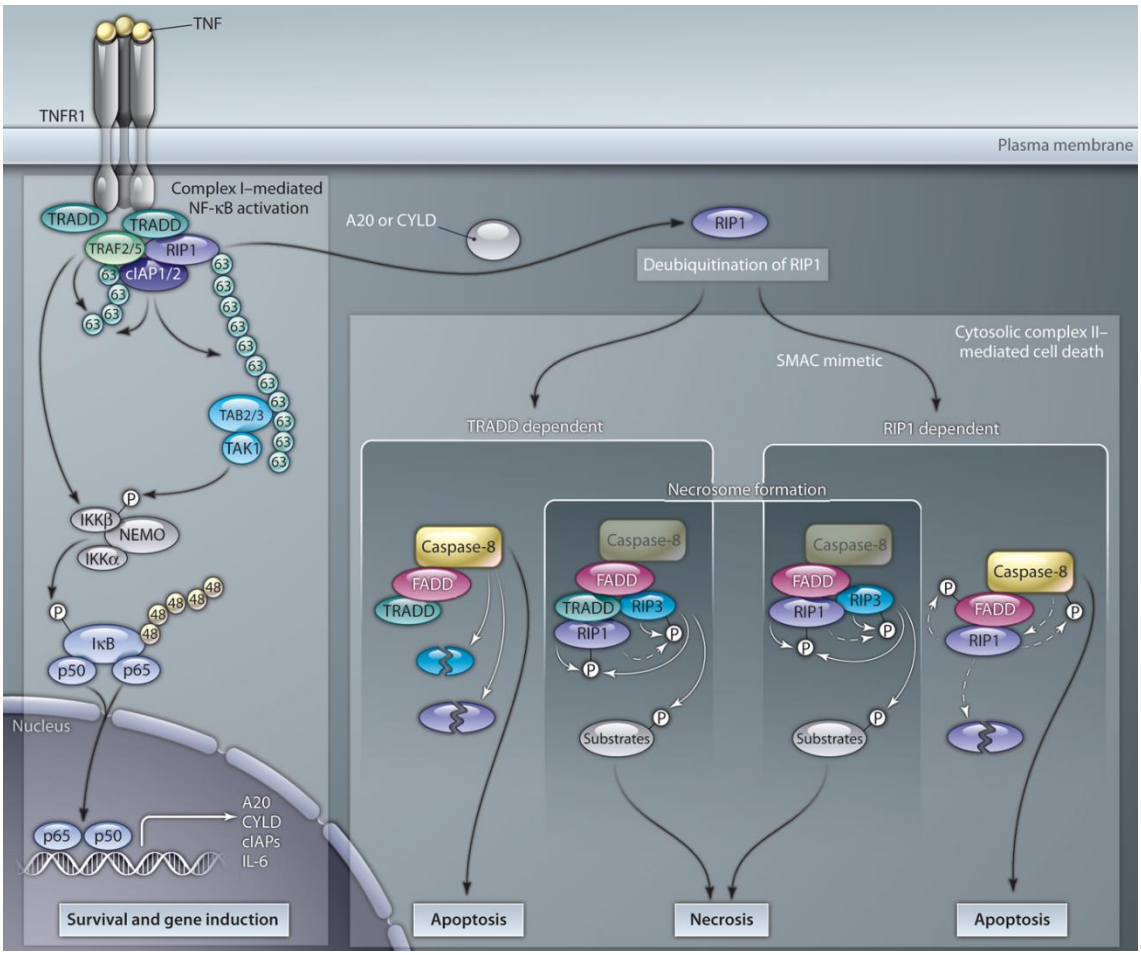

Figure 4. RIP1-mediated multimodal signaling events downstream of TNFR1. Upon binding to TNF-α, the cytoplasmic death domain of trimerized TNFR1 recruits a membrane-associated complex, named complex I, which comprises TRADD, RIP1, TRAF2, cIAP1, cIAP2 and the complex LUBAC. In complex I, RIP1 is polyubiquitinated, which mediates the recruitment and activation of TAK1 and inhibitor of κB (IκB) kinase (IKK) complex by ubiquitin binding. The phosphorylation and ubiquitin proteasome system-mediated degradation of IκB leads to activation of NF-κB. In contrast, alternative cytosolic complexes, complex IIa or complex IIb can also be formed. Complex IIa includes the adaptor protein FADD, caspase-8 and RIP1 and mediates the activation of caspase-8 and consequently apoptosis. When the activation of caspase-8 is inhibited, RIP1 kinase is activated and binds to RIP3 to form complex IIb, initiating necroptosis (Vandenabeele P et al, 2010).

More recently, mixed lineage kinase like (MLKL) has been reported as a key player in the necroptotic process. It appears that necrosome assembly leads to recruitment of MLKL (Conrad M et al, 2016). MLKL consists on a carboxy-terminal pseudokinase domain that is connected to an amino-terminal four-helix bundle (4HB) domain by a two-helix linker. This 4HB domain is the cell death executioner domain that is kept in an inactive state by the pseudokinase domain. Moreover, MLKL is phosphorylated by RIP3 at Thr357 and Ser358 that are located in the pseudokinase domain, thus allowing MLKL oligomerization and migration to the plasma membrane (Cai Z et al, 2014; Chen X et al, 2014; Hildebrand JM et al, 2014). The precise mechanism by which MLKL induces membrane rupture is controversial, with some reports implicating disruption of calcium or sodium ion channels (Cai Z et al, 2014; Chen X et al, 2014), and others showing direct binding to membrane phospholipids and disruption of membrane integrity (Dondelinger Y et al, 2014).

Importantly, under specific cellular contexts, increased levels of RIP3 or overexpression of a RIP3 phospho-mimetic mutant can trigger necroptosis in the absence of RIP1, suggesting RIP3 as the master regulator of necroptotic cell fate (Moujalled DM et al, 2013; Upton JW et al, 2010; Zhang DW et al, 2009).

Finally, the mitochondrial protein phosphoglycerate mutase family member 5 (PGAM5) was also suggested to interact with necrosome components. In fact, this protein was initially thought to recruit RIP1/RIP3 to the mitochondria to induce cell necroptosis (Wang Z et al, 2012). However, now it is known that the influence of PGAM5 on RIP1/RIP3 recruitment is very low. Moreover, PGAM5 appear to have a necroptosis protective function, both *in vitro* and *in vivo*, possibly due to its ability to promote PTEN-induced putative kinase 1 (PINK1)-dependent mitophagy of damaged mitochondria (Lu W et al, 2016). In addition, overproduction of ROS has also been described as a possible contributing factor in some cellular contexts (Degterev A et al, 2005; Zhang DW et al, 2009). Indeed, RIP3 can activate

multiple metabolic enzymes, thus increasing energy metabolism-associated ROS production (Zhang DW et al, 2009).

3.1.3. Necroptosis and inflammation

Necrosis is generally considered a proinflammatory type of cell death when compared to apoptosis. During necrosis, the intracellular constituents, or DAMPs, are released to the extracellular space where innate immune cells such as macrophages are located, thus promoting inflammation (Moriwaki K et al, 2013). Necroptosis, similar to necrosis, is also thought to result in the release of DAMPs into the extracellular space, including molecules with cytokine-like properties such as IL-1, ATP, high-mobility group protein B1 (HMGB1) and heat shock proteins, thus implicating necroptosis as a highly inflammatory type of cell death (Davidovich P et al, 2014) (Figure 5).

The effect of necroptotic cell death in inflammation is now a topic of strong research. For instance, studies using mice with epidermis-specific FADD deletion demonstrated that these animals presented spontaneous necroptosis of keratinocytes and developed severe inflammatory skin disease. The development of this inflammatory phenotype was dependent on RIP3-mediated necroptosis, since RIP3 deficiency fully prevented keratinocyte necroptosis and skin inflammation (Bonnet MC et al, 2011). Other studies reported that mice with epidermal keratinocyte restricted caspase-8 ablation developed severe inflammation, suggesting that caspase-8 deficiency triggered inflammation by a mechanism that involves p38-dependent upregulation of pro-caspase-1, resulting in inflammasome-mediated release of IL-1β (Kovalenko A et al, 2009). Interestingly, TLR4 signaling pathway appears to be involved in HMGB1 secretion from macrophages by a mechanism dependent on IL-1R-associated kinase 4 (IRAK4), indicating that HMGB1 might be released during TLR4-mediated necroptosis (Wang et al, 2010). Additionally, it has also

been proposed that TLR signaling contributes to the severe inflammatory response in ischemia-reperfusion injury models, indicating that, in this case, inflammation may be induced by DAMPs released by necroptotic cells (Wu et al, 2010).

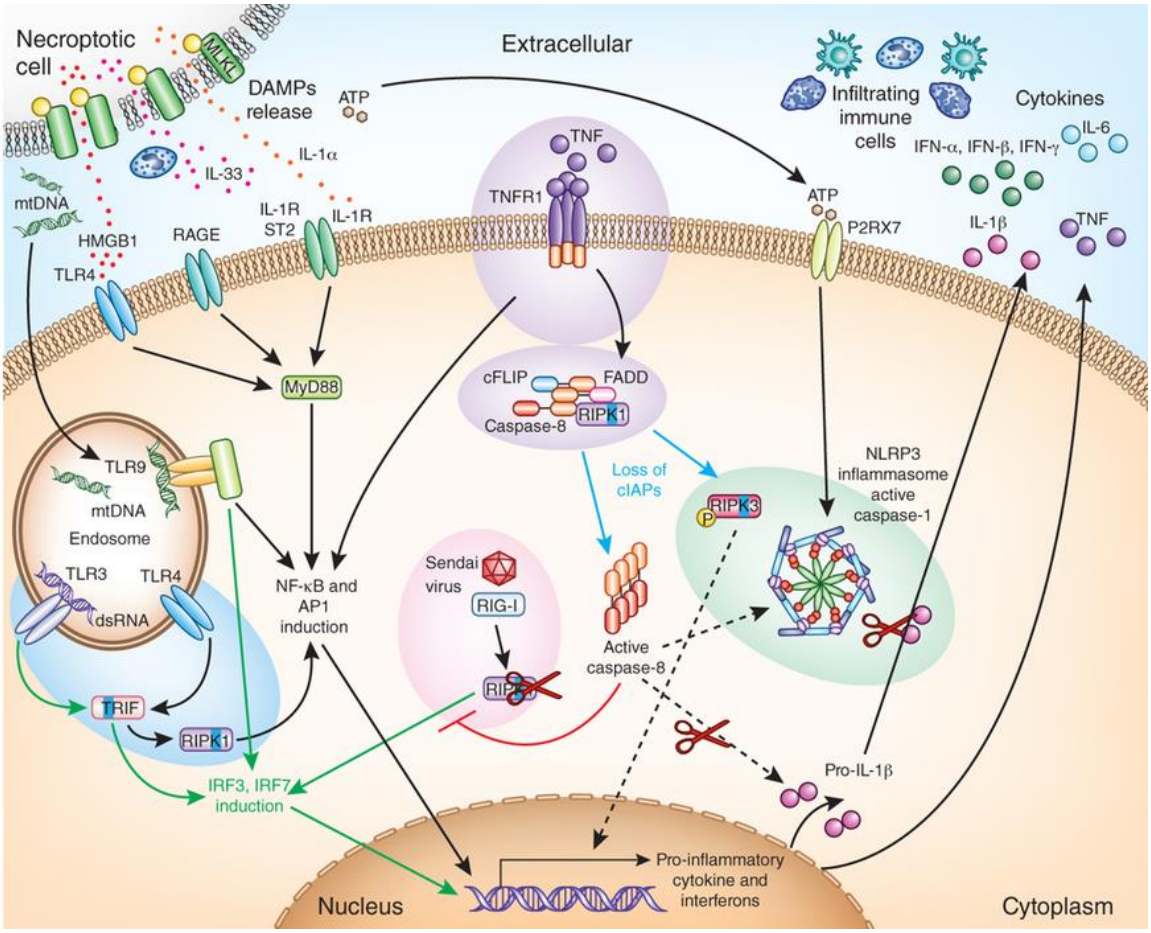

Figure 5. Necroptotic cells release proinflammatory mediators. Membrane rupture, an initial step of necroptosis, leads to the release of intracellular DAMPs to the extracellular space where innate immune cells are located, thus activating them to elicit a secondary inflammatory response. This inflammatory response is dependent on MyD88 and NF-κB signaling: HMGB1 via TLR4 and RAGE; IL-1 and IL-33 via IL-1R. For instance, HMGB1-TLR4 can be internalized into the endosome to initiate two different TRIF-dependent pathways: the first, a RIP1-dependent pathway, leading to NF-κB-induced proinflammatory cytokine production; and the second that involves the induction of interferon regulatory transcription factors (IRFs) and production of IFN-β. In addition, TNF-α triggers NF-κB signaling that is regulated by RIP1. Loss of cIAPs can lead to TNF- α-induced phosphorylation of RIP3

or activation of caspase-8. Phosphorylation of RIP3 can promote formation of the NLRP3 inflammasome, which activates caspase-1 generating mature IL-1β, or proinflammatory cytokine production (Silke J et al, 2015).

The role of RIP1 and RIP3 in animal models of human diseases has been highly associated with necroptosis. However, accumulating evidence indicates that RIP1 ubiquitination, deubiquitination and phosphorylation are the key events that determine whether a cell survive and activate an inflammatory response, or die through apoptosis or necroptosis (Christofferson DE et al, 2010; O'Donnell MA et al, 2011). In this regard, RIP1 was shown to have an important role in modulating NF-κB activation (Ting AT et al, 1996). Further, RIP1 was also implicated in TLR-mediated inflammation. After TLR3 and TLR4 activation, RIP1 modulates the downstream intracellular response, which involves two parallel pathways mediated by MyD88 and TRIF (Cusson-Hermance N et al, 2005; Meylan E et al, 2004) (Figure 5).

Other studies have shown that RIP1 kinase has an important role in regulating the inflammatory response in primary cell types such as macrophages and dendritic cells. In dendritic cells, deletion of caspase-8 results in increased production of proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-12, which is facilitated by RIP1 kinase activity (Moriwaki K et al, 2014), suggesting that caspase-8 is an important suppressor of the inflammatory response. Also, macrophages lacking cIAP1, cIAP2 and X-linked IAP (XIAP) secreted elevated levels of cytokines and chemokines, such as TNF-α and IL-6, which involves a mechanism that depends on RIP1 and RIP3, but not MLKL (Wong WW et al, 2014). Importantly, RIP1 knock-out mice die at birth due to severe systemic inflammation, whereas knock-in of RIP1 kinase-dead are viable and healthy, indicating that RIP1 has indeed a third function beyond its NF-κB-inducing activation and kinase function in necroptosis (Berger SB et al, 2014; Kaiser WJ et al, 2014).

Recently, it was also reported that RIP1 and RIP3 can regulate the inflammasome activation. In fact, caspase-8 deficiency in dendritic cells enhanced TLR4-induced formation and activation of the NLRP3 inflammasome by a mechanism dependent on RIP1, RIP3, MLKL and PGAM5, thus resulting in increased expression of mature IL-1β. Interestingly, these effects appear to be independent of necroptosis (Vince JE et al, 2012). Similarly, in IAP-competent cells, caspase-8 attenuates the assembly and function of NLRP3 inflammasome primed by TLR4 or TLR2 engagement, and the consequent IL-1β production is largely dependent on both RIP1 kinase activity and RIP3 (Kang TB et al, 2013).

3.1.4. Necroptosis in disease

The physiological relevance of necroptosis has been demonstrated in a variety of paradigms. In particular, necroptosis appears as a critical event in the pathogenesis of several inflammatory diseases, including neurodegenerative diseases. Indeed, increased expression of RIP1 and RIP3, two critical necroptosis mediators, is observed in many pathological conditions (Roychowdhury S et al, 2013; Vitner EB et al, 2014).

Necroptosis is involved in the pathogenesis of pancreatitis (He S et al, 2009), chronic inflammation of gut (Gunther C et al, 2011; Welz PS et al, 2011), skin inflammation (Bonnet MC et al, 2011), as well as lung (Rodrigue-Gervais IG et al, 2014), kidney (Tristão VR et al, 2012), heart (Smith CC et al, 2007), liver (Afonso MB et al, 2015; Takemoto K et al, 2014) and hematopoietic system injuries (Roderick JE et al, 2014). Importantly, in all cases, necroptosis inhibition resulted in disease improvement.

Regarding the CNS, other studies reported that necroptosis plays a critical role in the pathogenesis of cell death during traumatic brain injury, ischemic stroke, and neonatal hypoxia-ischemia brain injury. The link between necroptosis and neuronal damage has been suggested by studies demonstrating a protective effect of necroptosis inhibition in these

diseases. Indeed, in traumatic brain injury, mice administered with RIP1 kinase inhibitor necrostatin-1 (Nec-1) had reduced brain damage and improved motor and cognitive performance. In addition, Nec-1 also reduced brain neutrophil infiltration and suppressed microglial activation (You Z et al, 2008). Similarly, in a mouse model of transient focal cerebral ischemia, Nec-1 markedly decreased the infarct volume (Degterev A et al, 2005). Moreover, Nec-1 blocked RIP1/RIP3 interaction in neurons of a mouse model of ischemia brain injury. Importantly, necroptosis inhibition by Nec-1 also suppressed IL-1β, IL-6 and TNF-α expression, and NF-κB activation, thus highlighting the relevance of necroptosis inhibition to attenuate neuroinflammation (Northington FJ et al, 2011).

Inhibition of RIP1 kinase activity by Nec-1 was also protective against excitotoxicity in a primary rat cortical culture (Li Y et al, 2008) and in a mouse hippocampal cell line (Xu X et al, 2007). In addition, RIP3 deficiency alleviated the loss of mouse hippocampal neurons after stimulation with TNF-α (Liu S et al, 2014), as well as the onset and progression of disease in a transgenic mouse model of HD (Vandenabeele P et al, 2010; Zhu S et al, 2011), suggesting that necroptosis inhibition may have a beneficial effect in these conditions. Studies using co-cultures of primary motor neurons with astrocytes showed that astrocytes compromise neuronal survival in a RIP1 kinase- and MLKL-dependent manner. Importantly, Nec-1 rescues the death of motor neurons, indicating RIP1 inhibition as an important therapeutic strategy in ALS therapeutics (Re DB et al, 2014).

The contribution of necroptosis to neurodegeneration has been recently questioned by several authors that interpret microglia necroptosis as a strategy for preserving neurons. More specifically, it was shown that caspase-8 inhibition in mixed cerebellar cultures of primary neurons, astrocytes, and microglia prevented LPS-induced neuronal loss (Fricker M et al, 2013).

The contribution of necroptosis to inflammation is currently the focus of much attention. Of particular importance, caspase-8 is predominantly expressed in the microglial

lineage and defects in its activation have been associated with inflammation by engaging the necroptotic machinery (Wallach D et al, 2014). In this respect, a recent study showed elevated levels of inactive caspase-8 in MS patients, suggesting that a defect in caspase-8 activity in microglia in MS cortical lesions may activate necroptosis and promote inflammation (Ofengeim D et al, 2015). The same authors demonstrated that RIP1 and RIP3 were present at higher levels in an amyloid-like conformation in microglia, thus confirming necroptosis activation. Importantly, microglia necroptosis appears to promote inflammation through the release of TNF-α, which in turn further activates necroptosis in oligodendrocytes, thus contributing to disease progression (Ofengeim D et al, 2015).

3.1.5. Targeting necroptosis

RIP1 inhibitors

In 2005, a phenotypic screen for small molecules that inhibit TNF-induced necrotic cell death in human monocytic U937 cells identified the first RIP1 kinase inhibitor, Nec-1, which is an allosteric inhibitor of RIP1, stabilizing a specific inactive conformation of the kinase domain (Degterev A et al, 2008; Takahashi N et al, 2012). Nec-1 has increased specificity for RIP1, while it has no effect on RIP2 and RIP3, two RIP proteins with 33% sequence identity in the kinase domain. Therefore, Nec-1 is considered a potent necroptosis inhibitor (Degterev A et al, 2008). However, this molecule is a far-from-ideal drug, having a very short *in vivo* half-life and non-specific activity against indoleamine 2,3-dioxygenase (IDO), an enzyme also involved in the inflammatory response (Vandenabeele P et al, 2013). Importantly, it has been shown that Nec-1 protected mice and rats against neurodegenerative conditions, such as AD, HD, and stroke, as well as retinal degeneration and inflammatory diseases (Degterev A et al, 2005; Degterev A et al, 2008; Jouan-Lanhouet S et al, 2014). Other studies have also demonstrated that Nec-1 analog, Nec-1 stable (Nec-

1s), is > 1000-fold more selective than Nec-1, in addition to increased potency as necroptosis inhibitor (Degterev A et al, 2005; You Z et al, 2008). This molecule can prevent against TNF-induced lethality in a mouse model of systemic inflammatory response syndrome (SIRS) (Northington FJ et al, 2011). By contrast, Nec-1 inactive (Nec-1i) showed to be 100x less effective than Nec-1 and 10x less potent than Nec-1 and Nec-1s, and it is often used as an inactive control in studies using Nec-1 to exclude nonspecific off-target effects inherent to inhibitors. Recently, also the anti-leukemic agents and breakpoint cluster region-abelson leukemia (BCR-ABL) inhibitors ponatinib and pazopanib were indicated as inhibitors of both RIP1 and RIP3 proteins (Fauster A et al, 2015; Najjar M et al, 2015). Indeed, ponatinib and pazopanib inhibit RIP1- and RIP3-dependent cell death as well as transcription of TNF-α, thus indicating their cytoprotective and anti-inflammatory properties (Fauster A et al, 2015). Furthermore, fusion of the scaffold of ponatinib and Nec-1s generated a hybrid molecule, named PN10, a highly potent and selective RIP1 inhibitor, which are able to protect against TNF-induced SIRS *in vivo* (Najjar M et al, 2015).

RIP3 inhibitors

Among the years, the recognition that necroptosis could be activated independently of RIP1 and the observation that RIP3 knockout mice are viable led to the development of RIP3 inhibitors. Mandal et al. have shown that three selective small compounds GSK'840, GSK'843 and GSK'872 are able to inhibit RIP3-dependent necroptosis with high specificity by interacting with RIP3 to activate caspase-8 (Mandal P et al, 2014). Importantly, these compounds are able to prevent LPS-induced death of mouse macrophages, as well as cell death induced by TNF-α plus zVAD-fmk and poly(I:C)-triggered death of interferon-β-sensitized cells *in vitro*. However, these RIP3 kinase inhibitors have an unexpected caspase-independent cytotoxicity (Mandal P et al, 2014).

MLKL inhibitors

The first compound reported to inhibit MLKL was necrosulfonamide (NSA). This compound was used as a probe to identify MLKL as a downstream target of RIP3. This was confirmed by a study where NSA does not affect RIP3-dependent phosphorylation of MLKL. Later studies revealed that NSA can effectively block necroptosis induced by TNF-α plus zVAD-fmk in fibroblasts though interacting with MLKL (Sun L et al, 2012). However, NSA only acts on human MLKL and cannot be used to study necroptosis in mouse models. In addition, also a new class of MLKL inhibitors based on “compound 1” (GW806742X or SYN-1215) was developed and recently described to target the pseudokinase domain of MLKL. However, compound 1 also binds to RIP1 and inhibits its kinase activity, thus failing at implicating MLKL in necroptosis, due to its lack of specificity (J Silke, personal communication).

Despite of the efforts made so far, no inhibitors of necroptosis are available for clinical use. Therefore, the development of new molecules that potentially modulate this type of cell death are highly demanded.

4. Objectives

Here, we propose a new *in vitro* model to study necroptosis based on the murine BV2 microglial cell line. We aimed at identifying new inhibitors of necroptosis and further explore the signaling pathways involved. Additionally, we will also investigate if modulation of this type of cell death contributes to attenuate neuroinflammation, highlighting the potential use of necroptosis inhibitors to treat neurodegenerative diseases.

The main objectives of this study were to:

1. Implement a robust model to evaluate microglia necroptosis *in vitro*;
2. Screen a library of forty-one newly synthetized small molecules that potentially inhibit necroptosis;
3. Identify a lead molecule and characterize its *in vitro* activity;
4. Explore the signaling pathways involved in lead activity, namely necroptosis and inflammatory pathways.

II. MATERIALS AND METHODS

1. Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, necrostatin-1 (Nec-1) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO, USA). Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) pan caspase inhibitor was from Enzo Life Sciences (Farmingdale, NY, USA). Two libraries of twenty small molecules potentially inhibitors of necroptosis were obtained through a collaboration with Professor Carlos Afonso, from the Bioorganic Chemistry group within iMed.ULisboa.

2. Cell culture

The murine BV2 cell line was cultured in *Roswell Park Memorial Institute 1640* (RPMI) medium (GIBCO® Life Technologies, Inc.; Grand Island, USA), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (GIBCO) and 1% GlutaMAX™ (GIBCO). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Throughout the experiments, the culture media was replaced by a supplemented mixture without FBS, composed of RPMI, 1% penicillin/streptomycin, 1% insulin, transferrin, selenium and fibronectin (ITSF) (GIBCO), and 1 mg/mL bovine serum albumin (BSA) (GIBCO), next refered to as RPMI/ITSF.

3. Cell treatment

3.1. Optimization of an *in vitro* model of microglia necroptosis

BV2 cells were plated in 96-well plates at $1x10^4$ cells/well. Twenty-four hours after cell plating, media was removed and replaced with fresh RPMI/ITSF containing 100 ng/mL LPS for additional 24 h. After that, cells were pre-treated with 30 µM Nec-1 for 1 h, followed by 25 µM zVAD-fmk for additional 24 h. BV2 cells without any treatment were used

as control. Cell viability and cell death were analyzed by standard methods as detailed below.

3.2. Screening of small molecules for necroptosis inhibition

BV2 cells were plated in 96-well plates at $7x10^3$ cells/well. Twenty-four hours after cell plating, media was removed and replaced with fresh RPMI/ITSF containing 30 µM of each compound in test, 25 µM zVAD-fmk, zVAD-fmk plus 30 µM Nec-1 or compound for additional 24 h. BV2 cells without any treatment were used as control. Cell viability and cell death were analyzed by standard methods as detailed below.

3.3. EC_{50} determination

To determine the dose-response curve and EC_{50} value of the lead compound, BV2 cells were plated in 96-well plates at $7x10^3$ cells/well. Twenty-four hours after cell plating, media was removed and replaced with fresh RPMI/ITSF containing Oxa12 at different concentrations (0.1, 0.5, 1, 2.5, 5, 10, 30 and 50 µM) plus 25 µM zVAD-fmk. Following 24 h of compound exposure, cell viability was evaluated using standard methods as detailed below. The EC_{50} value was determined with GraphPad Prism v.5.00 (GraphPad Software, San Diego, CA, USA) using the log (inhibitor) *versus* response function. To determine Oxa12-associated toxicity, the same procedure was used but with a different range of concentrations (1, 2.5, 5, 10, 30, 50, 100 and 150 µM) without zVAD-fmk. In all cases, DMSO was used as control.

4. Evaluation of cell death and viability

Cell viability was measured using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's

instructions. This homogenous, colorimetric method determines the number of viable cells in cytotoxicity assays. The assay is composed of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron coupling reagent phenazine methosulfate (PMS). MTS is bioreduced by cells into a formazan product that can be measured spectrophotometrically at 490 nm in a plate reader. The conversion of MTS into the formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells, and is directly proportional to the number of viable cells in culture. In brief, cell culture supernatants were replaced by 100 µL of MTS/PMS (19:1) solution prepared in fresh RPMI/ITSF, and cells incubated at 37°C for 30 min. Changes in absorbance were measured at 490 nm using GloMax® Multi Detection System (Sunnyvale, CA, USA).

General cell death was evaluated using the lactate dehydrogenase (LDH) Cytotoxicity Detection Kit^{PLUS} (Roche Diagnostics GmbH, Manheim, Germany), according to the manufacturer's instructions. The LDH assay measures membrane integrity as a function of the amount of cytoplasmic LDH released into the medium that can be quantified by a coupled enzymatic reaction. In the first step, LDH catalyzes the conversion of lactate to pyruvate via reduction of NAD^+ to NADH. In the second step, diaphorase uses NADH to reduce a tetrazolium salt (INT) to a red formazan product. Thus, the level of formazan is directly proportional to the amount of LDH released, which is indicative of cytotoxicity. Hence, 50 µL of supernatant from treated cells was transferred into a new 96-well plate and then incubated with 50 µL of assay substrate for 10 to 30 min, at room temperature, protected from light. Absorbance readings were measured at 490 nm, with 620 nm reference wavelength using a Bio-Rad Model 680 microplate reader.

5. Evaluation of necroptosis signaling pathways

BV2 cells were plated in 6-well plates at $4x10^5$ cells/plate. Twenty-four hours after cell plating, media was removed and replaced with fresh RPMI/ITSF containing 100 ng/mL LPS for additional 24 h. After that, cells were pre-treated with 30 µM Nec-1 for 1 h, followed by 25 µM zVAD-fmk for additional 5 h. To evaluate Oxa12 potential in modulating necroptosis, cells were treated with zVAD-fmk, zVAD-fmk plus Nec-1, or zVAD-fmk plus Oxa12 for 24 h. BV2 cells without any treatment were used as control.

5.1. Total protein extraction

For total protein isolation, adherent cells were collected directly in nonyl phenoxypolyethoxylethanol (NP-40) lysis buffer (1% NP-40, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 1 mM dithiothreitol (DTT), and 1X proteases and phosphatases inhibitors), followed by sonication and centrifugation at 3200 *g* during 10 min at 4°C. Total protein extracts were recovered and stored at - 80 ºC.

Protein concentration was determined by the colorimetric Bradford method using the Bio-Rad Protein Assay reagent (Bio-Rad), according to the manufacturer's instructions. BSA (Sigma-Aldrich) was used as standard, and absorbance measurements were performed at 595 nm using Model 680 microplate reader (Bio-Rad). Protein concentrations were interpolated from the BSA standard curve.

5.2. Soluble and insoluble fractions

Necrosome formation was evaluated by the enrichment of necroptosis mediators in the insoluble proteome of BV2 cells. Adherent cells were collected in phosphate-buffered saline/ethylenediaminetetraacetic acid (PSB/EDTA), centrifuged at 600 *g* for 5 min at 4°C, and the pellet homogenized in NP-40 lysis buffer. Whole-cell lysates were then rotated for 30 min at 4°C, followed by centrifugation at 16000 *g* for 20 min at 4°C. Supernatants were

removed and used as the soluble fraction. To remove carryovers, the pellet was washed with NP-40 lysis buffer and further centrifuged at 16000 *g* for 10 min at 4°C. Then, the pellet was resuspended in urea-SDS buffer (8 M urea, 3% sodium dodecyl sulfate (SDS) in NP-40 lysis buffer) followed by sonication. Lysates were spun again at 16000 *g* for 20 min at 4°C, and the supernatant removed and used as the insoluble fraction. Protein concentration was determined using the bicinchoninic acid (BCA; Thermo Fisher Scientific, Inc.) assay for soluble and insoluble fractions, according to the manufacturer's recommendations.

5.3. Western blot analysis and densitometric analysis

Steady-state protein production was determined by Western blot analysis. Briefly, 40-50 µg of total protein extracts and 20 µg of soluble and insoluble protein fractions were denatured, electrophoretically resolved on 8% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes. Uniform protein loading and transfer was confirmed by transient staining with 0.2% Ponceau S (Merck, Darmstadt, Germany). Next, nonspecific binding sites were blocked with a 5% milk solution in Tris-buffered saline (TBS) for 1 h. Membranes were then incubated overnight at 4°C with primary rabbit antibodies reactive to RIP1, RIP3, Akt, p-Akt (Ser473) and NF-κB p65 (#7881; #135170; #8312; #7985; #372 from Santa Cruz Biotechnology; CA, USA), MLKL (#M6697; Sigma Aldrich), p-MLKL (Ser358) and p-NF-κB p65 (Ser536) (#196436; #131109 from Abcam; Cambridge, UK), p-p38 (Thr180/Tyr182) (#9211 from Cell Signaling; MA, USA); and with primary mouse monoclonal antibodies reactive to JNK, p-JNK (Thr183/Tyr185), p38α/β and IκBα (#7345; #6254; #7972; #371 from Santa Cruz Biotechnology) and p-IκBα (Ser32/36) (#9246 from Cell Signaling). Membranes were then washed three times with TBS containing 0.2% Tween 20 (TBS-T), and incubated with anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) for 2 h at room temperature. After rinsing three times with TBS-T, the immunoreactive complexes were visualized by chemiluminescence with

Immobilon™ Western (Milipore) or SuperSignal West Femto substrate (Thermo Fisher Scientific, Inc.). β-actin (AC-15; Sigma-Aldrich) was used as loading control. Densitometric analysis was performed with the Image Lab software Version 5.1 Beta (Bio-Rad).

6. Evaluation of inflammatory mediators

To evaluate transcript levels and secretion of inflammatory mediators, BV2 cells were plated in 12-well plates at $8x10^5$ cells/well. Twenty-four hours after cell plating, media was removed and replaced with fresh RPMI/ITSF containing 25 µM zVAD-fmk, 30 µM Nec-1, 30 µM Oxa12, or no addition, and zVAD-fmk plus Nec-1 or Oxa12 for additional 24 h. Adherent cells were used for total RNA extraction and qRT-PCR analysis, while cell culture media was used for ELISA.

6.1. Total RNA extraction and quantitative Real-Time PCR

Total RNA was extracted from BV2 cells with TRIzol™ reagent (Invitrogen, Grand Island, USA), according to the manufacturer's protocol. RNA was quantified using a Qubit™ 2.0 fluorometer (Invitrogen).

Real-time PCR (RT-PCR) was performed to determine the expression level of COX2, IL-1β, IL-6, NLRP3 and TNF-α. Reverse Transcription reactions were performed using total RNA and NZY Reverse Transcriptase (NZYTech, Lisbon, Portugal), according to the manufacturer's instructions. RT-PCR reactions were performed using specific primers and SYBR Green PCR master mix (Thermo Fisher Scientific, Inc.). Triplicate reactions were run per sample using a 7300 Real-Time PCR System, and data was acquired and analyzed using 7000 System sequence Detection Software, version 1.2.3 (Applied Biosystems, Thermo Fisher Scientific, Inc.). The relative amounts of each gene were calculated based on the standard curve normalized to the level of hypoxanthine-guanine

phosphoribosyltransferase (HPRT) and expressed as fold-change from control cells. Primer sequences are presented in Table 1.

Table 1. Primer sequences used to amplify indicated cDNAs.

Primer	Forward (5'-3')	Reverse (5'-3')
COX2	CAGCCAGGCAGCAAATCCTT	AGTCCGGGTACAGTCACACT
HPRT	GGTGAAAAGGACCTCTCGAAGTG	ATAGTCAAGGGCATATCCAACAACA
IL-1β	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
IL-6	GACGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
NLRP3	AGAGCCTACAGTTGGGTGAAATG	CCACGCCTACCAGGAAATCTC
TNF-α	AGGCACTCCCCCAAAAGATG	TGAGGGTCTGGGCCATAGAA

6.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Sandwich ELISA kits (PeproTech, London, UK) were used to determine TNF-α concentration in BV2-treated cells culture media. The kit contains 96-well plates that were coated with a capture antibody specific for TNF-α overnight at room temperature. The next day, the liquid was removed and wells washed 4x with washing buffer (0.05% Tween-20 in PBS). Blockage of non-specific binding was performed by adding blocking buffer (1% BSA in PBS) for 1 h at room temperature, followed by the same cycle of washes. At this point, 100 µL of BV2 cell supernatants were added to each well and incubated for 2 h at room temperature. 4 washes were performed as before. The detection antibody was then added to each well and incubated for 2 h at room temperature, followed by 4 washes as before. Finally, avidin peroxidase was added and incubated during 30 min at room temperature, followed by 4 washes as before. This step allows the linking of peroxidase over a strepdavidin-biotin bridge to the antibody-TNF-α-antibody sandwich structure. Conversion of

the colorless substrate into a soluble colored product was achieved by adding peroxidase substrate solution, which is enzymatically processed into a blue colored product. Color intensity is proportional to the quantity of TNF-α contained in each sandwich structure. Therefore, samples were incubated at room temperature until the development of blue color was visually detectable (± 30 min), and then absorbance was read at 450 nm, with 590 nm reference wavelength using a Bio-Rad Model 680 microplate reader. TNF-α concentration (pg/mL) was calculated from standard curves.

7. Microscopy

BV2 cells morphology was evaluated by phase-contrast microscopy using a Primo Vert microscope (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Images were acquired under 100x magnification using an AxioCam 105 Color camera with the ZEN lite 2012 (both from Carl Zeiss MicroImaging GmbH).

8. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) of at least three independent experiments. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's test. Analyses and graphical presentation were performed with the GraphPad Prism software version 5 (GraphPad Software, Inc., San Diego, CA, USA). The statistical significances were achieved when $p < 0.05$.

III. RESULTS

zVAD-fmk induces necroptosis in BV2 microglia cells

Recent studies have demonstrated that primary microglia undergo RIP1/RIP3-dependent necroptosis after treatment with LPS or TLR ligands, when caspases are inhibited (Fricker M et al, 2013; Kim SJ et al, 2013). Importantly, other studies also showed that treatment with the pan caspase inhibitor zVAD-fmk alone induces necroptosis in L929 cells, by a mechanism that depends on autocrine production of TNF-α (Christofferson DE et al, 2012; Wu YT et al, 2011). In the present study, we propose a new *in vitro* model for the study of microglia necroptosis, based on the murine BV2 microglial cell line. We evaluated necroptosis activation after 24 h treatment with LPS, a well-known TLR4 agonist, followed by incubation with the pan caspase inhibitor, zVAD-fmk, for additional 24 h. Importantly, we also determined if zVAD-fmk alone was capable of inducing necroptosis in BV2 cells.

As depicted in Figure 6, high levels of cell death were detected in BV2 cells after treatment with zVAD-fmk alone, as evaluated by MTS metabolism and LDH activity assays ($p < 0.001$). Incubation with LPS prior to zVAD-fmk further decreased BV2 cells viability in approximately 30%, as revealed by MTS ($p < 0.001$). Importantly, addition of Nec-1, a RIP1-specific kinase inhibitor, fully reverted cell death to control levels in all conditions tested ($p < 0.001$), thus implicating RIP1-dependent necroptosis as the death mechanism (Fig. 6). These results indicate that zVAD-fmk alone is capable of inducing necroptosis in BV2 cells. However, co-incubation with LPS appears to potentiate or accelerate the cell death mechanism, suggesting the involvement of necroptosis in an inflammatory context.

Others have also reported that primary microglia treated with LPS after caspase inhibition show an increase in RIP1 and RIP3 total protein levels, the major effectors of necroptosis (Fricker M et al, 2013). We confirmed these results as BV2 treated with LPS for 24h presented increased RIP1 and RIP3 total protein levels, suggesting that LPS predisposed cells to necroptosis induction (Fig. 7). As expected, LPS/zVAD-fmk co-

incubation further increased RIP1 and RIP3 total levels when compared to LPS alone, indicating necroptosis activation in these conditions. This is in accordance with MTS/LDH data.

Surprisingly, Nec-1 treatment induced a marked increase of RIP1 and RIP3 total protein levels (Fig. 7). Therefore, we decided to investigate the accumulation of both proteins in the insoluble proteome, as necroptosis activation requires the assembly of a detergent insoluble amyloid-like complex called necrosome.

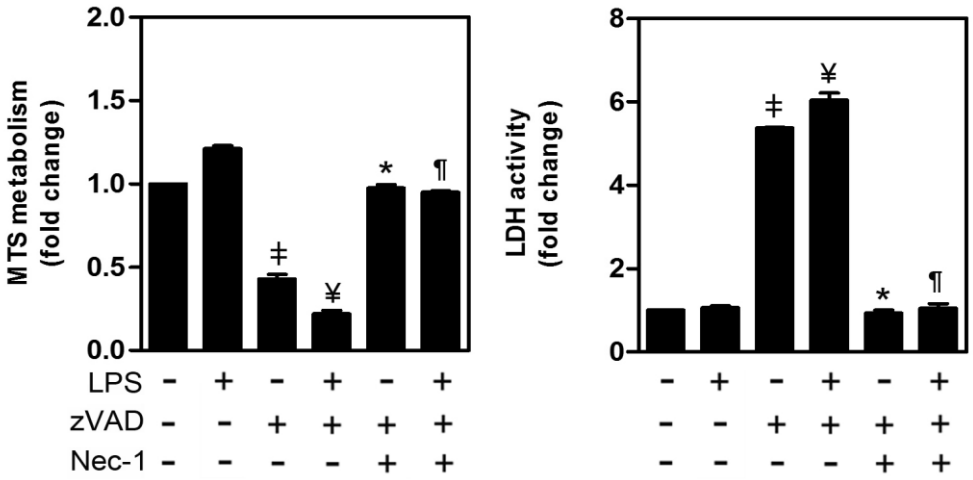

Figure 6. BV2 microglial cells undergo necroptosis when exposed to LPS/zVAD-fmk or zVAD-fmk alone. BV2 cells were pre-treated with 100 ng/mL LPS for 24 h and then exposed to the pan-caspase inhibitor zVAD-fmk (25 µM) for additional 24 h. Cell metabolic activity was determined by MTS metabolism and cell membrane integrity by LDH activity assays. Nec-1 (30 µM) was added 1 h before zVAD-fmk. Results are presented as the mean value ± SEM of three independent experiments performed in duplicates and normalized to control cells (‡ $p < 0.001$ vs. control; ¥ $p < 0.001$ vs. LPS; * $p < 0.001$ vs. zVAD-fmk; ¶ $p < 0.001$ vs. LPS/zVAD-fmk).

zVAD-fmk promotes necrosome assembly in BV2 microglia cells

RIP1 and RIP3 assembly into the necrosome is crucial for necroptosis activation (Li J et al, 2012). Thus, we evaluated the presence of RIP1/RIP3 in detergent insoluble protein fractions of BV2 cells upon zVAD-fmk treatment with or without LPS. As shown in Figure 8, RIP1 and RIP3 were sequestered in the insoluble fraction in cells pre-treated with LPS for 24 h and then incubated with zVAD-fmk for additional 5 h, thus confirming functional necrosome assembly and, consequently, necroptosis activation (Fig. 8A). Cells solely treated with zVAD-fmk had no RIP1 and RIP3 in this fraction, suggesting that at this time-point zVAD-fmk is still not capable of inducing necroptosis (Fig. 8A). Curiously, more than 5 h of LPS/zVAD-fmk resulted in complete loss of plasma membrane integrity and presence of cellular debris (data not shown).

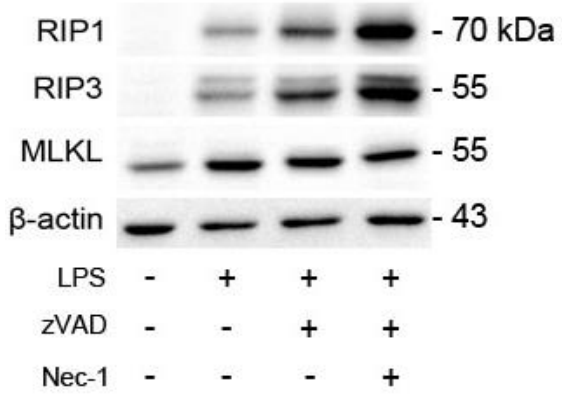

Figure 7. LPS exposure increases RIP1 and RIP3 total protein levels. BV2 cells were pre-treated with 100 ng/mL LPS for 24 h and then exposed to the pan-caspase inhibitor zVAD-fmk (25 μM) for additional 24 h. Nec-1 (30 μM) was added 1 h before zVAD-fmk. Total protein lysates were prepared for Western blot analysis of RIP1, RIP3 and MLKL. β-actin was used as loading control.

To corroborate MTS and LDH assay data and confirm necroptosis activation in that same conditions, we incubated BV2 cells with zVAD-fmk for 24 h. The results showed

the presence of RIP1 and RIP3 in the insoluble fraction, thus confirming necrosome assembly, indicating that zVAD-fmk induces necroptosis in BV2 cells after 24 h (Fig. 8B). Importantly, addition of Nec-1 abolished RIP1 and RIP3 sequestration in the insoluble fraction, both in zVAD-fmk and LPS/zVAD-fmk-treated cells, thus confirming that the marked increase in total protein levels had no functional consequence in terms of induction of necroptosis (Fig. 8). Taking these results into account, BV2 cells exposed to zVAD-fmk are a robust *in vitro* model of microglia necroptosis, which is fully reverted when RIP1 kinase activity is inhibited by Nec-1.

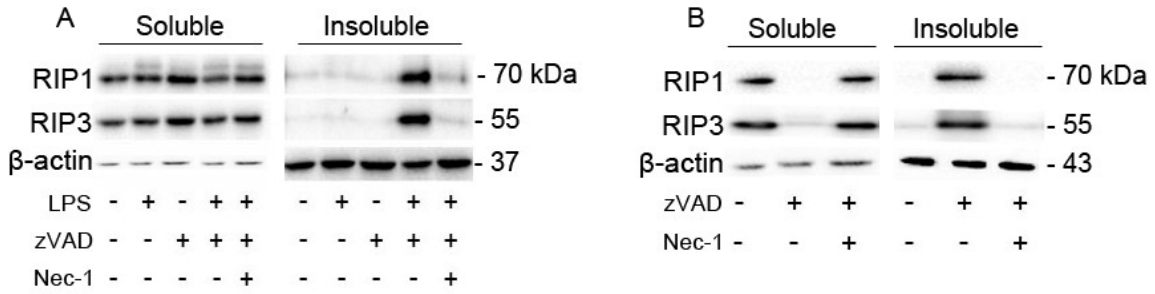

Figure 8. zVAD-fmk treatment only induces necrosome assembly at 24 h of incubation. (**A**) BV2 cells were pre-treated with 100 ng/mL LPS for 24 h and then exposed to the pan-caspase inhibitor zVAD-fmk (25 µM) for additional 5 h. Nec-1 (30 µM) was added 1 h before zVAD-fmk. (**B**) BV2 cells were co-treated with zVAD-fmk (25 µM) plus Nec-1 (30 µM) for 24 h. Detergent soluble and insoluble fractions were prepared for Western blot analyses of RIP1 and RIP3. β-actin was used as loading control.

Screening for potential inhibitors of necroptosis

Tool compounds blocking necroptosis have been developed since 2005; however, to date, no necroptosis inhibitors are in clinical use. In this regard, the identification of new molecules with potential for inhibiting this type of cell death is strongly needed.

Here, we used our established BV2 cell line *in vitro* model to screen two in-house chemical libraries of new compounds, designed as potential inhibitors of necroptosis. The first family of twenty compounds tested had no impact in zVAD-fmk-induced cell death in this cell line, as observed by MTS metabolism (Fig. 9).

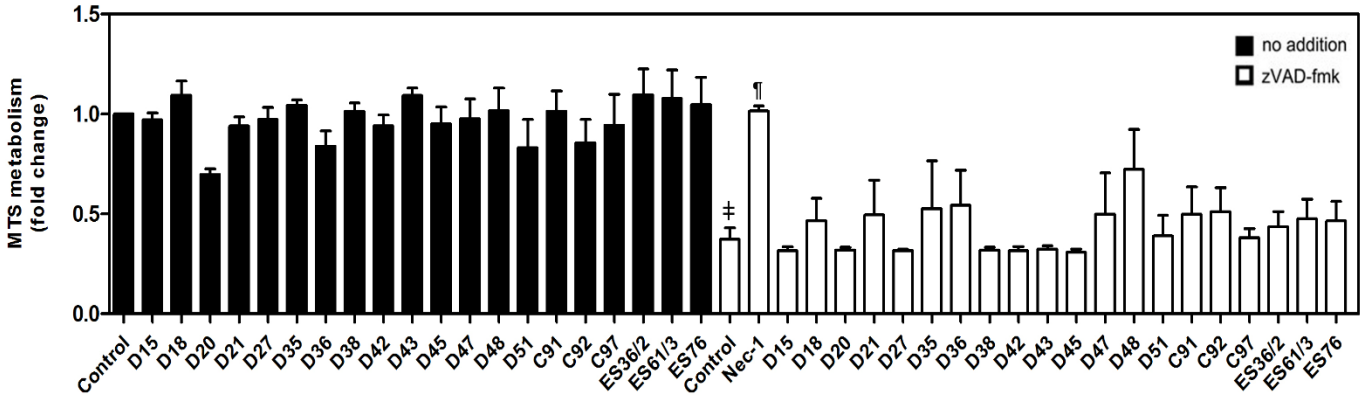

Figure 9. The first chemical library of twenty compounds does not modulate necroptosis in BV2 microglial cell line. BV2 cells were incubated with zVAD-fmk (25 µM); zVAD-fmk (25 µM) plus Nec-1 (30 µM); compounds (30 µM) or zVAD-fmk (25 µM) plus compounds (30 µM) for 24 h. Cell metabolic activity was determined by MTS metabolism. The results are presented as the mean value ± SEM of three independent experiments performed in duplicates and normalized to control BV2 cells (‡ $p < 0.001$ vs. control no addition; ¶ $p < 0.001$ vs. control zVAD-fmk).

However, we were able to identify one hit within the second family of twenty-one compounds (Oxa1-21). Oxa12 significantly reduced zVAD-fmk-mediated cell death, as observed by MTS and LDH assays ($p < 0.05$) (Fig. 10). Notably, treatment of BV2 cells with Oxa12 increased cell viability ($p < 0.05$) and decreased membrane permeabilization ($p < 0.05$) (Fig. 10) by approximately 40%, relatively to zVAD-fmk alone.

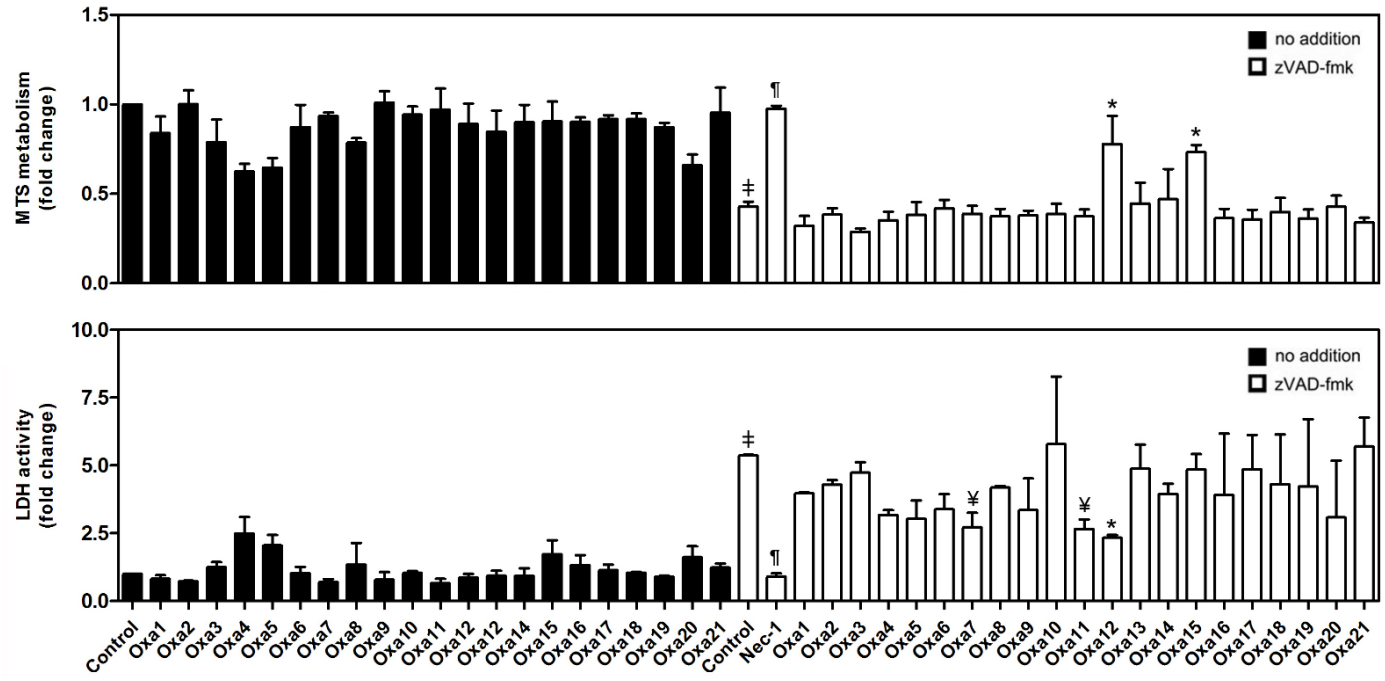

Figure 10. Oxa12 modulates necroptosis in the BV2 microglial cell line. BV2 cells were incubated with zVAD-fmk (25 μM); zVAD-fmk (25 μM) plus Nec-1 (30 μM); compounds (30 μM) or zVAD-fmk (25 μM) plus compounds (30 μM) for 24 h. Cell metabolic activity was determined by MTS metabolism and cell membrane integrity was determined by LDH activity. Data are presented as the mean value ± SEM of three independent experiments performed in triplicates and normalized to control BV2 cells (ǂ $p < 0.001$ vs. control no addition; ¶ $p < 0.001$ vs. control zVAD-fmk; ¥ $p < 0.01$ vs. control zVAD-fmk; * $p < 0.05$ vs. control zVAD-fmk).

Oxa12 modulates necroptosis in the BV2 microglial cell line

To further characterize Oxa12 activity, drug toxicity was assessed by determining its half maximal inhibitory concentration (IC_{50}). We incubated BV2 cells with different concentrations of Oxa12 ranging from 1 to 150 μM, and determined that this compound alone did not induce any cytotoxicity in BV2 cells, even at high concentrations (IC_{50}>130 μM) (Fig. 11A). Additionally, we also determined its half maximal effective concentration (EC_{50}) for inhibiting necroptosis using concentrations from 0.1 to 50 μM. Our results showed that

Oxa12 was effective at inhibiting zVAD-fmk-induced necroptosis, with an EC_{50} of 1.422 µM (Fig. 11B).

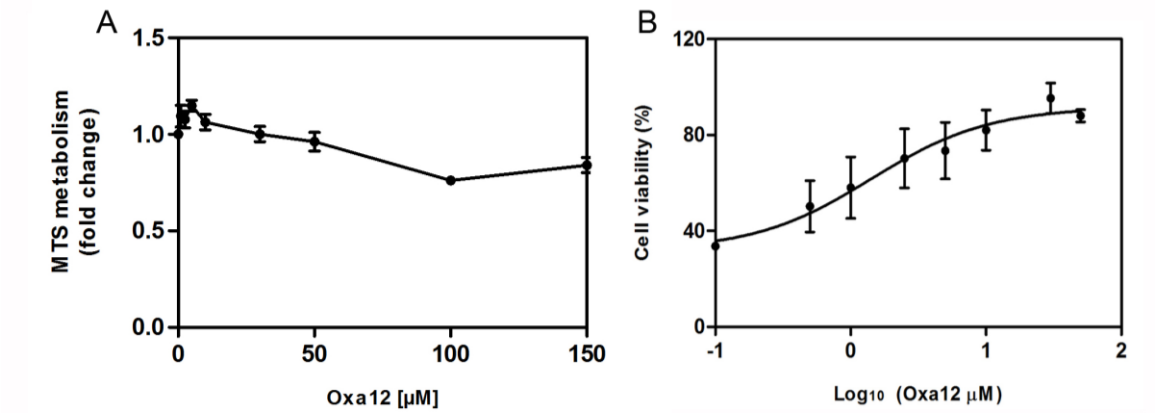

Figure 11. Oxa12 is effective at inhibiting zVAD-fmk-induced necroptosis without toxicity. (**A**) BV2 cells were incubated with different concentrations of Oxa12 (1, 2.5, 5, 10, 30, 50, 100 and 150 µM) for 24 h. (**B**) BV2 cells were incubated with different concentrations of Oxa12 (0.1, 0.5, 1, 2.5, 5, 10, 30, and 50 µM) plus zVAD-fmk (25 µM) for 24 h. Cell metabolic activity was determined by MTS metabolism. The results are presented as the mean value ± SEM of three independent experiments performed in duplicates and normalized to vehicle control (DMSO).

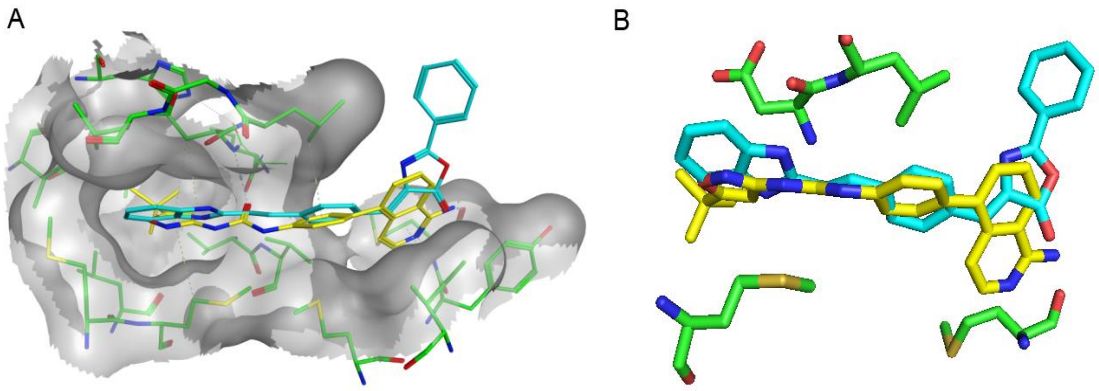

Figure 12. *In silico* molecular docking calculations for Oxa12. (**A**) Optimal poses obtained inside RIP1 active site (grey) for Oxa12 (represented in stick model and colored blue) compared with

crystallographic ligand 1-aminoisoquinoline inhibitor (PDBID: 4NEU) (yellow). (**B**) Oxa12 and 4NEU co-crystallized inhibitor interacting with Asp156, Leu157, Met67 and Met95. Docking calculations were performed using the X-ray structure obtained for RIP1 complexed with 1-aminoisoquinoline inhibitor at resolution of 2.57 Å, PDBID: 4NEU, by the GOLD 5.2 software.

Finally, to explain the observed biological activity and to get insight into the mechanism of action of Oxa12 at the molecular level, we performed *in silico* molecular docking calculations for Oxa12 inside the RIP1 kinase domain using the X-ray structure obtained for this enzyme complexed with 1-aminoisoquinoline type II kinase inhibitor (Harris PA. ACS Med. Chem. Lett 2013, 4(12)). The docking studies were conducted by Prof. Rita Guedes from the Medicinal Chemistry group within iMed.ULisboa. Our results showed that without any constrain (e.g. a covalent bond), Oxa12 is occupying a pose similar to the co-crystallized inhibitor, with the phenyl rings from both compounds almost superposed, thus suggesting a similar interaction (Fig. 12A). However, Oxa12 is slightly rotated when compared with the crystallographic ligand, being close to Asp156, Leu157, Met67 and Met95, which may enable important hydrogen bonds and π interactions. Importantly, Oxa12 showed increased interaction distances in comparison to the crystallographic inhibitor (Fig. 12B).

Oxa12 inhibits necroptosis in the BV2 microglial cell line

In order to determine the effect of Oxa12 in the necroptotic signaling pathway, we co-incubated cells with zVAD-fmk plus Oxa12 for 24 h and evaluated both necrosome assembly and MLKL S358 phosphorylation in detergent insoluble fractions. MLKL phosphorylation at T357/S358 residues is mediated by RIP3 during necroptosis, leading to

MLKL oligomerization, thus being an excellent marker of necroptosis commitment (Ofengeim D et al, 2015; Wang H et al, 2014).

As depicted in Figure 8 and Figure 13, we confirmed that at this time-point, incubation with zVAD-fmk significantly increased the levels of RIP1 and RIP3 in the insoluble fraction ($p < 0.05$), thus suggesting functional necrosome assembly and necroptosis activation. Additionally, the levels of MLKL S358 phosphorylation were also significantly increased ($p < 0.05$) (Fig. 13). Nec-1 incubation totally abrogated both conditions ($p < 0.05$). Of note, Oxa12 prevented all necroptosis-associated events, including RIP1/RIP3 functional necrosome assembly and MLKL S358 phosphorylation ($p < 0.05$) (Fig. 13).

Finally, we analyzed BV2 morphology after zVAD-fmk treatment with and without Nec-1 and Oxa12. As expected, both compounds reverted zVAD-fmk-induced cell morphology (Fig. 14). Taken together, these results implicate Oxa12 as a strong inhibitor of necroptosis and a promising candidate molecule for targeting RIP1/3-driven pathophysiology.

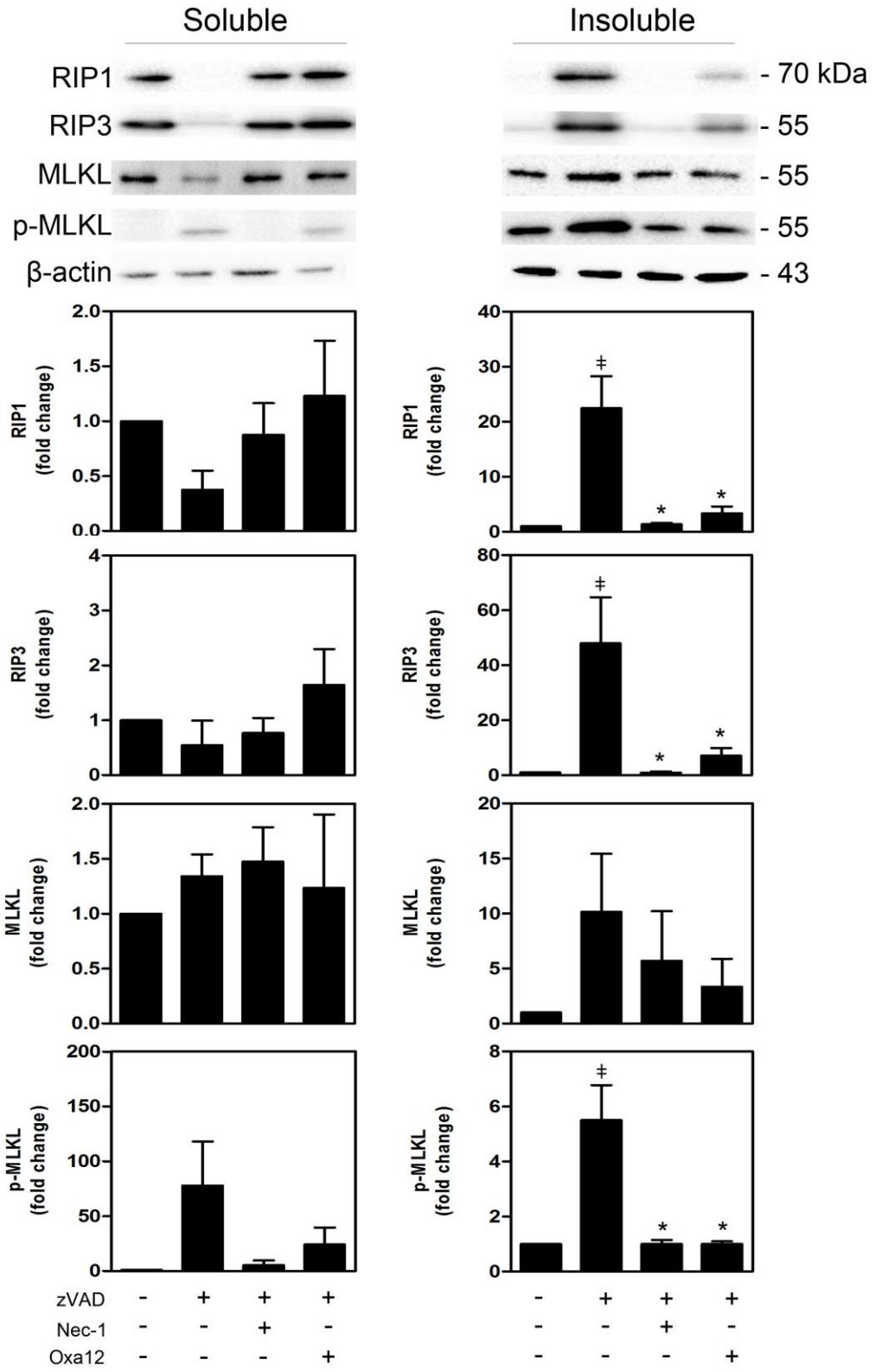

Figure 13. Oxa12 modulates necroptosis in a microglial cell line. BV2 cells were incubated with zVAD-fmk (25 µM); zVAD-fmk (25 µM) plus Nec-1 (30 µM) or zVAD-fmk (25 µM) plus Oxa12 (30 µM)

for 24 h. Detergent soluble and insoluble protein fractions were prepared for Western blot analysis of RIP1, RIP3, MLKL and p-MLKL. Representative immunoblots are presented with the respective densitometric analysis. β-actin was used as loading control. Values are expressed as mean ± SEM of three independent experiments (ǂ $p < 0.05$ vs. control; * $p < 0.05$ vs. zVAD-fmk).

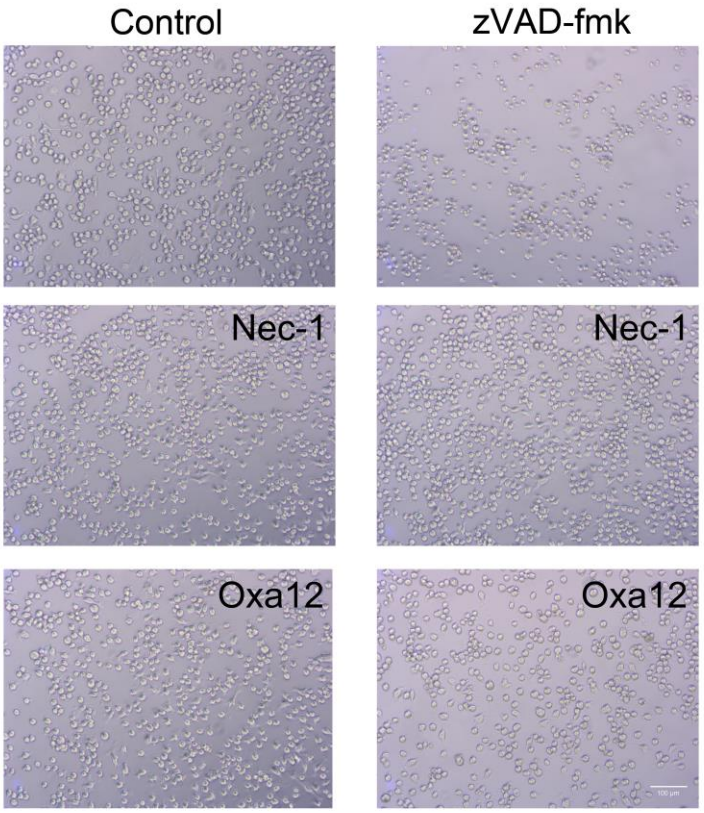

Figure 14. Oxa12 reverts zVAD-fmk-induced cell morphology. BV2 cells were incubated with zVAD-fmk (25 µM); Nec-1 (30 µM); zVAD-fmk (25 µM) plus Nec-1 (30 µM); Oxa12 (30 µM) or zVAD-fmk (25 µM) plus Nec-1 (30 µM) for 24 h. Microscopy images were taken at 100x with a Primo Vert microscope.

Oxa12 reduces TNF-α gene expression and secretion

Necroptosis is a proinflammatory type of cell death, due to the release of intracellular components called DAMPs to the extracellular space where innate immune cells are located, thus enhancing inflammation (Moriwaki K et al, 2013). Therefore, after

confirming that Oxa12 inhibited necroptosis in BV2 cells, we hypothesized that this inhibition may also contribute to decreased inflammation.

In this respect, other studies have reported that zVAD-fmk-induced necroptosis is dependent on the production and autocrine secretion of TNF-α (Wu YT et al, 2011). Thus, we assessed TNF-α secretion levels by ELISA as well as expression of proinflammatory genes, including *COX2, IL-1β, IL-6*, *NLRP3* and *TNF-α* by qRT-PCR.

The results showed that zVAD-fmk treatment for 24 h significantly enhanced TNF-α secretion levels, with an increase of approximately 2-fold relative to control cells ($p < 0.05$). In contrast, Nec-1 fully reverted TNF-α secretion to control levels ($p < 0.05$) and more importantly, Oxa12 significantly reduced its levels by approximately 1.5-fold ($p < 0.05$) (Fig. 15). All together, these findings suggest that autocrine secretion of TNF-α is a key step of zVAD-fmk-induced necroptosis. Oxa12 induces a marked decrease in TNF-α secretion, probably by inhibiting the necroptotic signaling pathway.

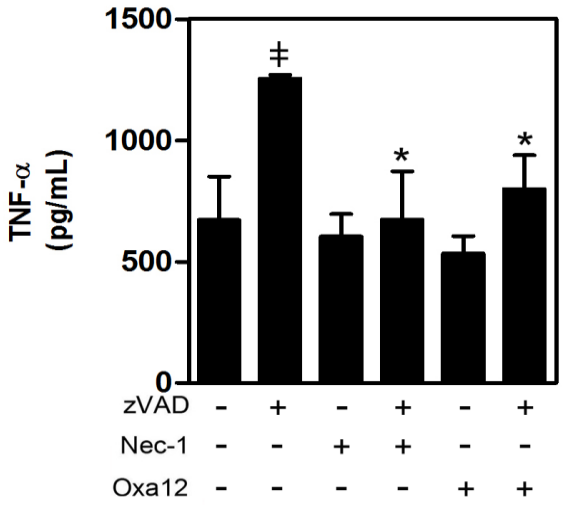

Figure 15. Oxa12 decreases TNF-α secretion levels. BV2 cells were incubated with zVAD-fmk (25 μM); Nec-1 (30 μM), zVAD-fmk (25 μM) plus Nec-1 (30 μM); Oxa12 (30 μM), or zVAD-fmk (25 μM) plus Oxa12 (30 μM) for 24 h. Secreted TNF-α was measured with a murine TNF-α ELISA kit. Results are presented as mean ± SEM from three independent experiments, and expressed as absolute TNF-α levels (‡ $p < 0.05$ vs. control and * $p < 0.05$ vs. zVAD-fmk).

Regarding the transcription of proinflammatory genes, the results showed that exposure of BV2 cells to zVAD-fmk for 24 h resulted in a significant increase of *TNF-α* and *IL-1β* gene expression ($p < 0.001$), which was totally reverted upon Nec-1 incubation ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 16). Importantly, Oxa12 also reduced *TNF-α* gene expression by about 40% ($p < 0.05$), which was in accordance with the results obtained in the ELISA assay. Surprisingly, incubation with Oxa12 had an opposite effect in *IL-1β* expression ($p < 0.05$) increasing transcript levels more than 2-fold (Fig. 16). Relatively to *COX2* and *NLRP3*, the results did not show any significant variation (Fig. 16), and the expression levels of *IL-6* were barely detectable in all conditions tested.

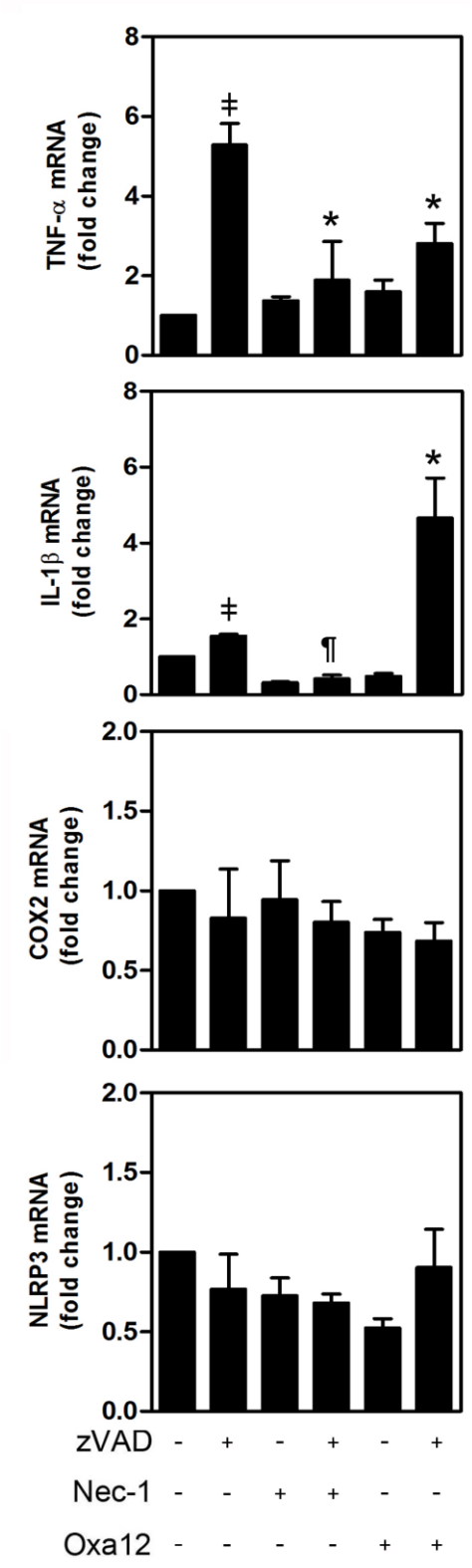

Figure 16. Oxa12 decreases TNF-α gene expression. BV2 cells were treated with zVAD-fmk (25 μM); Nec-1 (30 μM), zVAD-fmk (25 μM) plus Nec-1 (30 μM); Oxa12 (30 μM), or zVAD-fmk (25 μM)

plus Oxa12 (30 μM) for 24 h. mRNA levels of proinflammatory mediators (TNF-α, IL-1β, COX2 and NLRP3) were measured by RT-PCR. Results are expressed as mean ± SEM from three independent experiments (ǂ $p < 0.001$ vs. control; ¶ $p < 0.001$ vs. zVAD-fmk; * $p < 0.05$ vs. zVAD-fmk).

Oxa12 modulates zVAD-fmk-induced JNK and p38 MAPK signaling activation and increases IκB phosphorylation

To further dissect which inflammatory pathways Oxa12 specifically targets, we evaluated JNK (Thr183/Tyr185) and p38 (Thr180/Tyr182) phosphorylation, two important MAPK signaling pathways involved in inflammation (Kim EK et al, 2010). In addition, we also evaluated phosphorylation of protein kinase B, also known as Akt (Ser473). Indeed, others have shown that JNK and Akt, when activated, have important roles in necroptosis, where they are involved in the production and autocrine secretion of TNF-α (Christofferson DE et al, 2012). Importantly, phosphorylation of these proteins is strongly related with their activation (Wu YT et al, 2011). NF-κB activation, here evaluated by IκB (Ser32/36) phosphorylation, is also involved in inflammation and cell survival (Rubio-Perez JM et al, 2011). IκB phosphorylation leads to its own degradation and, consequently, the release and nuclear translocation of NF-κB where it exerts activity as a transcription factor.

As observed in Figure 17, incubation of BV2 cells with zVAD-fmk for 24 h induced a significant increase in JNK and p38 phosphorylation ($p < 0.01$ and $p < 0.001$, respectively), thus suggesting activation of these two signaling pathways. By contrast, Nec-1 fully reverted JNK and p38 phosphorylation to control levels ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 17). Importantly, Oxa12 significantly decreased both JNK ($p < 0.05$) (Fig. 17A) and p38 phosphorylation levels ($p < 0.001$) (Fig. 17B), when compared to zVAD-fmk-treated cells. Regarding Akt activation, no significant changes were observed in these experimental conditions (Fig. 18). Nevertheless, zVAD-fmk treatment appeared to reduce IκB phosphorylation ($p < 0.05$), which was counteracted by Nec-1 ($p < 0.05$). Addition of Oxa12

to the culture media also increased IκB phosphorylation ($p < 0.05$), thus suggesting NF-κB activation (Fig. 19).

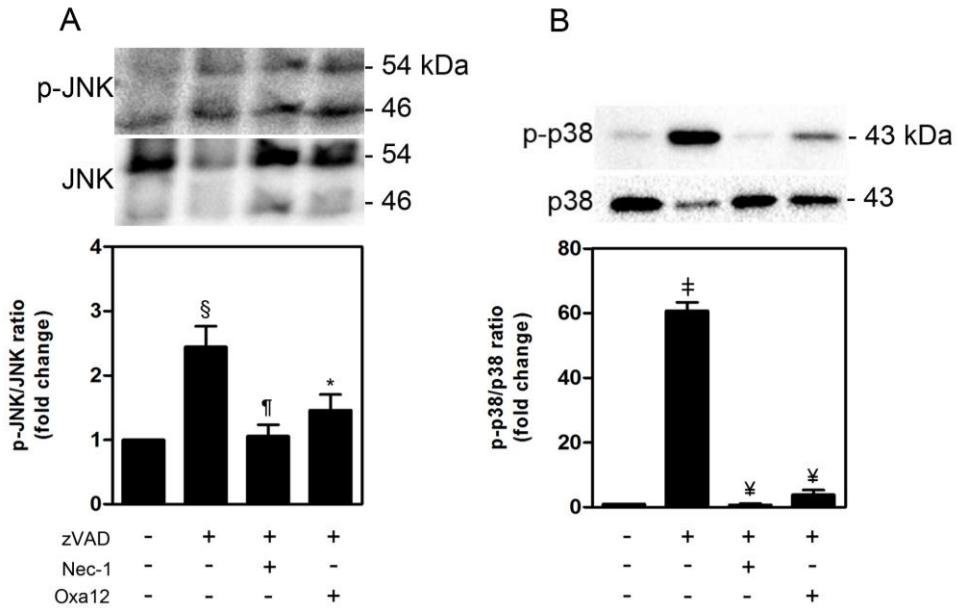

Figure 17. Oxa12 decreases zVAD-fmk-induced JNK and p38 MAPK activation. BV2 cells were treated with zVAD-fmk (25 μM), zVAD-fmk (25 μM) plus Nec-1 (30 μM) or zVAD-fmk (25 μM) plus Oxa12 (30 μM) for 24 h. (**A**) Representative immunoblots of p-JNK (Thr183/Tyr185) and total JNK are presented, together with the respective densitometric analysis of the p-JNK/JNK ratio. (**B**) Representative immunoblots of p-p38 (Thr180/Tyr182) and total p38 are presented with the respective densitometric analysis of the p-p38/p38 ratio. Values are expressed as mean ± SEM of three independent experiments (‡ $p < 0.001$ vs. control; ¥ $p < 0.001$ vs. zVAD-fmk; § $p < 0.01$ vs. control; ¶ $p < 0.01$ vs. zVAD; * $p < 0.05$ vs. zVAD).

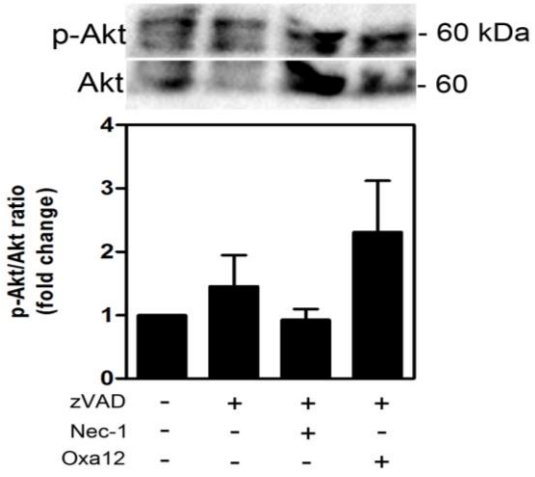

Figure 18. zVAD-fmk incubation does not affect Akt phosphorylation in BV2 cells. BV2 cells were incubated with zVAD-fmk (25 μM), zVAD-fmk (25 μM) plus Nec-1 (30 μM) or zVAD-fmk (25 μM) plus Oxa12 (30 μM) for 24 h. Representative immunoblots of p-Akt (Ser473) and total Akt are presented together with the

respective densitometric analysis of the p-Akt/Akt ratio. Results are expressed as mean ± SEM from three independent experiments.

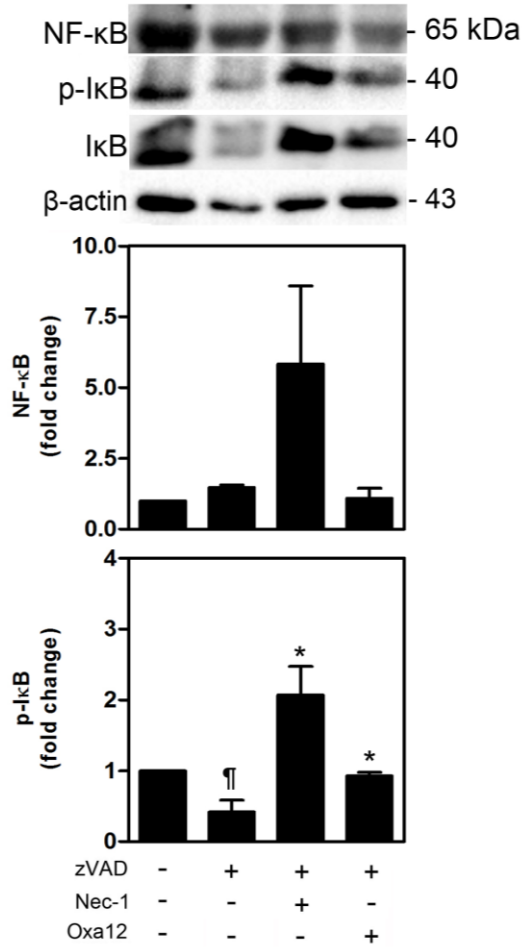

Figure 19. Oxa12 increases IκB phosphorylation levels when compared to zVAD-fmk-treated cells. BV2 cells were incubated with zVAD-fmk (25 μM), zVAD-fmk (25 μM) plus Nec-1 (30 μM) or zVAD-fmk (25 μM) plus Oxa12 (30 μM) for 24 h. Representative immunoblots of total NF-κB, p-IκB (Ser32/36) and total IκB are presented together with the respective densitometric analysis. β-actin was used as loading control. Results are expressed as mean ± SEM from three independent experiments (¶ $p < 0.05$ vs. control; * $p < 0.05$ vs. zVAD-fmk).

IV. DISCUSSION

Neurological disorders are an important cause of mortality and represent 12% of total deaths worldwide. The prevalence of neurodegenerative diseases, in particular, is expected to continue to enlarge, mostly due to the increase in the lifespan of the general population, which constitutes a major health economic challenge for Europe.

Neurodegenerative diseases are mainly characterized by progressive neurodegeneration, presence of abnormal protein assemblies, oxidative stress and neuroinflammation. Currently, the treatments available are ineffective. Therefore, new interventions that slow or stop neurodegeneration are an urgent, unmet need.

Although apoptotic cell death is frequently implicated in neurodegeneration, necroptosis has been recently described as a prominent player in neurodegenerative diseases pathobiology (Vandenabeele P et al, 2010). Necroptosis is a caspase-independent form of regulated cell death, executed via activation of RIP1 and RIP3, being negatively regulated by caspase-8 (Vercammen D et al, 1998). Neuroinflammation is also a pathological hallmark of neurodegenerative diseases, where microglia have a fundamental role regulating both innate and adaptive immune responses (Rubio-Perez JM et al, 2011).

Throughout the recent literature, the role of necroptosis has been studied in the pathogenesis of several diseases, with the results indicating necroptosis inhibition as an improvement factor (He S et al, 2009; Takemoto K et al, 2014). Regarding the CNS, most studies implicate necroptosis in neuronal death and refer inhibition of necroptosis as a beneficial event (Degterev A et al, 2005; Liu S et al, 2014; Northington FJ et al, 2011; Xu X et al, 2007). Conversely, other authors demonstrated that primary microglia treated with caspase inhibitors undergo necroptosis, and this mechanism has been interpreted as a strategy for preserving neurons (Fricker M et al, 2013). This raises the question whether protection against necroptotic microglial cell death might also be beneficial in the CNS given that necroptotic cell death often results in inflammation. Indeed, it was recently shown that in certain pathological conditions, such as MS, microglia present a strong defect in caspase-8

activation, which may promote inflammation through activation of necroptosis, thus contributing to the progression of the disease (Ofengeim D et al, 2015). In these cases, targeting the necroptotic machinery may be useful to attenuate microglial-mediated inflammation and cell death.

In this work, we used the murine BV2 microglia cell line as a new and robust *in vitro* model for screening potential modulators of microglia necroptosis. BV2 cells are derived from raf/myc-immortalized murine neonatal microglia and are the most frequently used cells to model primary microglia (Lund S et al, 2005).

Our results showed that BV2 cells undergo necroptosis after 24 h incubation with the pan caspase inhibitor, zVAD-fmk, which is in agreement with previous studies reporting that zVAD-fmk induces necroptosis in L929 cells (Chistofferson DE et al, 2012; Wu YT et al, 2011). However, pre-incubation with LPS accelerated or potentiated the death mechanism, thus implicating necroptosis in this inflammatory context. Indeed, we observed a decrease of ~30% in MTS metabolism when cells were pre-incubated with LPS for 24 h and, then, incubated with zVAD-fmk for additional 24 h, while no differences were observed in LDH activity. These results suggest that cell membrane permeabilization and leakage of intracellular components, such as LDH, may be an initial step in the necroptotic cascade. In contrast, ROS production and mitochondria dysfunction appears to contribute indirectly to late-stage necroptosis (Wang H et al, 2014; Chen X et al, 2014; Tait SW et al, 2013). In addition, we observed that, while LPS/zVAD-fmk co-treatment induces necrosome assembly after 5 h incubation, zVAD-fmk alone induces necrosome assembly only after 24 h.

LPS is a well-known TLR4 agonist that was already shown to induce RIP3-dependent necroptosis, after caspase inhibition (Fricker M et al, 2013). On the other hand, it is known that zVAD-fmk triggers the production of TNF-α at the transcriptional level, and subsequently the autocrine secretion of this cytokine, which in turn may activate TNFR to induce necroptosis (Wu YT et al, 2011). Thus, it was not surprising that zVAD-fmk alone

induced necroptosis at later time-points. In contrast, the RIP1-specific inhibitor, Nec-1, here used as a positive control of necroptosis inhibition, fully reverted cell death to control levels in all conditions tested, thus implicating RIP1-dependent necroptosis as the death mechanism. Additionally, the reversion of necrosome assembly induced by Nec-1 incubation confirmed the importance of this molecular platform as a cell death inducer in our model. Overall, BV2 cells exposed to zVAD-fmk represent a robust *in vitro* model of microglia necroptosis, along with full reversion of all necroptotic processes when RIP1 kinase activity is inhibited by Nec-1.

In 2005, a phenotypic screening for small molecules that inhibit TNF-induced cell death identified the first necroptosis inhibitor, Nec-1, which inhibits RIP1 kinase activity (Degterev A et al, 2005). Since then, several necroptosis inhibitors have been described, including RIP1, RIP3 and MLKL inhibitors. However, all compounds identified so far show several limitations. Nec-1, for instance, is highly effective in inhibiting necroptosis (EC_{50}=0.494 μM); however, it has inadequate pharmacokinetic properties including very short *in vivo* half-life of approximately 1 h, along with reduced solubility. In addition, it was recently shown that Nec-1 has non-specific activities against IDO, an enzyme involved in inflammation, as well as against serine/threonine-protein kinase 1 (PAK1) and protein kinase cAMP(PKA)-dependent catalytic subunit, indicating Nec-1 potential off-target activity (Jagtap PG et al, 2007; Vandenabeele P et al, 2013). Regarding RIP3 inhibitors, all compounds described so far present caspase-independent cytotoxicity, along with the ability to induce apoptosis (Mandal P et al, 2014). Finally, NSA, a known MLKL inhibitor, inhibits necroptosis by blocking human MLKL phosphorylation, but not the rodent homolog, invalidating pharmacological, pharmacokinetic and toxicity preclinical testing (Sun L et al, 2012). In sum, although tool compounds blocking necroptosis have been developed, no necroptosis inhibitors are in clinical use to date. Therefore, the discovery of new specific and potent pharmacologic inhibitors of necroptosis is of utmost importance.

In this study, we screened two libraries of new compounds from two different families that potentially modulate necroptosis. In the first family of compounds, none of the molecules were able to modulate necroptosis. However, in the second family of compounds, we identified one hit (Oxa12) that inhibits necroptotic cell death by approximately 40% relatively to zVAD-fmk-treated cells, being effective at low concentrations (EC_{50}=1,422 μM) and without cytotoxicity associated. Further, Oxa12 inhibited all necroptosis-associated events, including necrosome assembly and MLKL S358 phosphorylation, two important markers of necroptosis commitment. Importantly, the docking pose of Oxa12 inside RIP1 active site is similar to the co-crystallized 1-aminoisoquinoline inhibitor, suggesting its potential as necroptosis inhibitor.

To further characterize the mechanism of action of Oxa12 and because necroptosis is an inflammatory type of cell death, we hypothesized that necroptosis inhibition by Oxa12 could also result in decreased inflammation. Our results showed that BV2 cells exposed to zVAD-fmk presented increased levels of TNF-α gene expression and cytokine secretion. These findings were in accordance with previous studies reporting the production and autocrine secretion of TNF-α as a crucial factor in zVAD-fmk-induced necroptosis (Hitomi J et al, 2008; Wu YT et al, 2011). Moreover, zVAD-fmk also induced IL-1β gene expression, thus confirming the proinflammatory potential of necroptosis. Finally, no differences were detected in COX2 and NLRP3 transcription levels, which might be due to the involvement of IL-1β in the initiation of the inflammatory cascade, while COX2 and NLRP3 are mainly involved in later stages, being extensively regulated by other proteins and signaling cascades (Abderrazak A et al, 2015; Bakhle YS et al, 1996; Latz E et al, 2013).

Similarly to what happens with Nec-1 (Chistofferson DE et al, 2012), treatment of BV2 cells with Oxa12 also reduced TNF-α gene expression and cytokine secretion levels. Importantly, inhibition of necroptosis by Nec-1 suggests that RIP1 kinase activity is required for TNF-α production, as previously reported (Christofferson DE et al, 2012). Nec-1 also

reverted IL-1β gene expression being indicated as an anti-inflammatory molecule (Nikseresht S et al, 2015). In contrast, we cannot rule out that inhibition of necroptosis by Oxa12 results in increased inflammation through a compensatory mechanism, as we observed a marked increase in IL-1β gene expression after Oxa12 incubation. However, IL-1β processing and activation is dependent on NLRP3 inflammasome and caspase-1 activity. NLRP3 inflammasome assembly occurs in response to an inflammatory stimulus and leads to caspase-1 activation, which in turn mediates the cleavage of cytosolic pro-IL-1β into the mature and secreted proinflammatory cytokine (Baroja-Mazo A et al, 2014). Since we did not observe any modulation of NLRP3 gene expression, it is unlikely that Oxa12-mediated increase in IL-1β mRNA does translate into increased levels of secreted IL-1β. Still, further studies are needed to confirm these results.

To better understand which inflammatory pathways are specifically targeted by Oxa12, we evaluated the activation of two MAPK signaling pathways, JNK and p38, as well as the activation of Akt. Recent studies have reported that JNK activation plays an important role during necroptosis in L929 cells downstream to RIP1 kinase (Christofferson DE et al, 2012). Indeed, it has been demonstrated that zVAD-fmk induces activation of protein kinase C (PKC), which activates JNK, leading to the activation of transcription factor activating protein 1 (AP1) that, in turn, promotes TNF-α gene expression (Wu YT et al, 2011). Interestingly, it was also reported that RIP1 may interact with the E3 ubiquitin ligase EDD, and cIAP1, leading to JNK activation and consequently to specificity protein 1 (Sp1)-dependent transcription of TNF-α (Christofferson DE et al, 2012). Importantly, increased TNF-α transcription may translate into elevated levels of this cytokine, which could induce necroptosis through TNFR activation (Wu YT et al, 2011). Our results are in line with these studies, since exposure of BV2 cells to zVAD-fmk induced high levels of JNK activation. This activation, in turn, may be related with the increase observed in TNF-α gene expression and cytokine secretion levels. By contrast, Nec-1 and Oxa12 abolished both JNK activation and

TNF-α gene expression and cytokine secretion levels, thus confirming the involvement of this signaling pathway in zVAD-fmk-induced necroptosis.

Regarding p38, the results described so far are somehow contradictory. Some authors reported that this signaling pathway is not activated during necroptosis in L929 cells (Yu L et al, 2004). Conversely, others have shown that pharmacological inhibition of TNF-α-induced necroptosis in L929 cells resulted in p38 activation, as well as inhibition of JNK (Ye YC et al, 2011). Here, we show that treatment of BV2 cells with zVAD-fmk induces a marked increase in p38 phosphorylation, which is fully reverted after Nec-1 and Oxa12 incubation. These findings suggest activation of this signaling pathway during zVAD-fmk-mediated necroptosis in BV2 cells. In addition, it is well known that both JNK and p38 MAPK pathways are involved in stress responses and inflammation. Indeed, these kinases are activated by several inflammatory mediators in different cell lines (Dhillon AS et al, 2007). Thus, it is possible that the intracellular components released by necroptotic cells may induce JNK and p38 MAPK activation.

Furthermore, a recent study suggested that Akt is linked to necroptosis in L929 cells, where it plays a key role in mediating TNF-α synthesis (Wu YT et al, 2009). Inhibition of Akt protected L929 cells from TNF-α-induced necroptosis (Jin S et al, 2007). Indeed, in some conditions, inhibition of Akt and its downstream substrate mechanistic target of rapamycin (mTOR) reduces TNF-α-induced necroptosis and triggers autophagy instead. This study also showed that Akt and mTOR activation is not induced by TNF-α or zVAD-fmk alone, but it requires stimulation by both TNF-α and zVAD-fmk (Liu Q et al, 2014). In contrast, others have demonstrated that zVAD-fmk alone is indeed capable of inducing Akt activation, with this activation being dependent on Thr308 phosphorylation, while no alterations were observed in Akt Ser473 phosphorylation (McNamara CR et al, 2013). In our work, we evaluated Akt Ser473 phosphorylation as a marker of Akt activation, which may explain the

absence of significant differences between the conditions tested. Nevertheless, further studies are needed to confirm these results.

Finally, we determined NF-κB activation by evaluating IκB phosphorylation. Phosphorylation of IκB by IKK complex leads to its proteolysis and, consequently, to translocation of NF-κB dimers to the nucleus where they can induce the expression of target genes involved in inflammatory and pro-survival responses (Xiao G et al, 2001). Some studies have shown that inhibition of TNF-α-induced necroptosis may promote NF-κB activation (Wang D et al, 2013). Interestingly, mounting evidence disregards NF-κB signaling as a modulator of autocrine TNF-α expression upon zVAD-fmk exposure (Wu YT et al, 2011). Our results are in accordance with these findings, since zVAD-fmk treatment strongly reduced IκB phosphorylation levels. By contrast, both Nec-1 and Oxa12 further increased IκB phosphorylation levels, when compared to zVAD-fmk treatment. Therefore, it is possible that zVAD-fmk incubation induces a switch between a pro-survival pathway mediated by NF-κB and the activation of the necroptotic signaling, while Nec-1 and Oxa12 have the opposite effect.

Overall, we established a robust *in vitro* model of microglia necroptosis, based on the murine BV2 microglial cell line and identified a strong lead inhibitor of this type of regulated cell death. In addition, Oxa12 demonstrated to be efficient at decreasing TNF-α gene expression and secretion levels, as well as activation of important signaling pathways including JNK and p38 MAPK. Moreover, our compound is effective at switching a necroptotic to a pro-survival signaling pathway mediated by NF-κB activation. Thus, Oxa12 can now be considered a promising candidate molecule for targeting RIP1 and RIP3-driven pathologies.

V. CONCLUSION AND FUTURE PERSPECTIVES

In this work, we established a new *in vitro* model of microglia necroptosis, based on the murine BV2 cell line. We identified one lead compound (Oxa12) that strongly inhibits the necroptotic signaling pathway without cytotoxicity associated. In addition, Oxa12 decreases TNF-α gene expression and secretion levels, as well as JNK and p38 MAPK activation, while increases IκB phosphorylation levels. These results suggest that Oxa12 is capable of inducing a switch from a necroptotic pathway to a pro-survival signaling mediated by NF-κB.

Still, further studies are needed to determine which is the specific target of this compound in the necroptotic signaling pathway. Regarding this question, we are currently engaged in determining whether Oxa12 presents any off-target effects, which should be achieved by performing a chemical proteomic profile. Moreover, all the compounds tested in this work were dissolved in DMSO, which raises some concerns about their solubility. In this respect, we intend to evaluate Oxa12 aqueous solubility and, if necessary, optimize its molecular structure so that it can be used *in vivo*. Oxa12 pharmacodynamics, biodisponibility and half-life will be also ascertained.

Regarding the role of Oxa12 in inflammation, it is important to clarify if Oxa12-induced transcription of IL-1β does translate into increased levels of IL-1β secretion. Moreover, further studies are required to evaluate the role of Akt activation in necroptosis, as well as the effect of Oxa12 in this signaling pathway, which might be attained by evaluating Akt phosphorylation in other residues including Thr308. To deepen the knowledge of the role of JNK and p38 in zVAD-fmk-induced necroptosis, we will further study their downstream targets.

Finally, it would be also interesting to confirm the protective effects of Oxa12 in primary cultures of microglia and in co-cultures of primary microglia and neurons. We intend to determine if Oxa12-mediated inhibition of microglia necroptosis translates into an improvement of neuronal survival, which might point to an important therapeutic strategy for neurodegenerative diseases. Currently, there is no cure for neurodegenerative diseases and the treatments available are merely symptomatic. Therefore, the discovery of new interventions that slow or stop

neurodegeneration and neuroinflammation are an urgent, unmet need. In this regard, modulation of necroptosis may stand out as an unprecedented therapeutic strategy.

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